A STUDY OF THE ADENOVIRUS DNA-BINDING PROTEIN (DBP) : PREPARATION OF ANTIBODIES AGAINST PHOSPHOTYROSINE AND DBP AND THEIR USE IN INVESTIGATING TYROSINE PHOSPHORYLATION OF DBP

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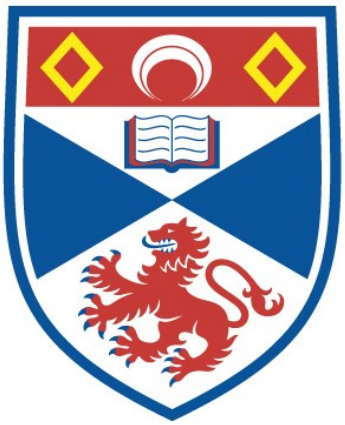
A Thesis Submitted for the Degree of MPhil
at the
University of St Andrews



1994

A Study Of The Adenovirus DNA-Binding Protein (DBP): Preparation Of Antibodies Against Phosphotyrosine and DBP and their use in investigating tyrosine phosphorylation of DBP

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April, 1993

This thesis is submitted for the degree of Master of Philosophy.

Declaration

I, Yuejiao Li, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

signed

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Acknowledgements

I would like to thank the following people—

Professor W. C. Russell, my supervisor for his guidance and encouragement throughout the course of this work;

Mr Ian R. Leith, my colleague for his continuous helping with practical work and providing purified DBP;

Dr A. Webster for her enthusiasm & willingness to give help and providing ^{32}P-labelled ssDNA probe;

Dr G. Kemp & Mr P. Talbot for helping with peptides synthesis;

Miss K.Spooner & Mr D.Young for helping with MAb techniques;

Mr T. Hanke for helping with protein expressing techniques;

Mr B. Blyth for his co-operative photographic services;

Mrs & Mr Anya Xiaocao for looking after my daughter Mingming when completing this work.

Dedication

To Mingming

Abstract

It has become clear that protein tyrosine phosphorylation plays a central role in cellular regulation and viral transformation. Adenovirus DNA-binding protein (DBP) is a multi-functional phosphoprotein and a previous study suggested that DBP is phosphorylated at a tyrosine residue (Russell et al, 1989). In order to investigate tyrosine phosphorylation of DBP, attempts were made to prepare polyclonal and monoclonal antibodies against phosphotyrosine (Ptyr) and DBP (adenovirus type2 DNA-binding protein) with success except in the case of the monoclonal antibody (MAb) against Ptyr (Part I). These antibodies were used in parallel to detect any Ptyr of DBP in an immunoassay (Part II). Anti-DBP polyclonal antibody was used in immunoprecipitation as well as western blotting and MAb to DBP was used in western blotting and immunofluorescence. Anti-DBP MAb was also used in characterizing the DNA-binding domain of DBP. Polyclonal anti-Ptyr antibody reacted well with phosphotyrosine-containing protein EGFR in immunofluorescence, immunoprecipitation and western blotting but failed to detect Ptyr in DBP in these assays, whereas a synthetic peptide DBPPT195, which consists of a putative tyrosine phosphorylation site of DBP [aa (amino acid) 195] and its surrounding residues (aa188-aa201), was shown to be phosphorylated in vitro. In order to obtain an unphosphorylated DBP and mutate the tyrosine (which was suggested to be phosphorylated) of DBP, an attempt to express DBP in E.coli was also made (Part III). The DBP gene

was inserted into pGEX-2T vector but the production of fusion protein in E.coli was very poor .

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Methods:

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Introduction

Three topics are reviewed here: 1. adenoviruses that encode the DBP protein; 2. protein phosphorylation that modulates the DBP protein; 3. the DBP protein that has been utilised with in this work.

1. Adenovirus

The adenoviruses are a group of DNA viruses that infect vertebrate cells and in humans cause mainly respiratory disease. These viruses lack envelopes and contain only DNA and proteins. They replicate and assemble their progeny in the nuclei of the infected cells, the viral polypeptides being synthesized in the cytoplasm and all transported to the nuclei. The virons are released via cell destruction (Ginsberg, 1984).

There are 47 serotypes of the adenoviruses which have been identified to date in humans. They share many common features including virion architecture, genome organisation, polypeptide composition and replicative cycles etc. On the basis of oncogenicity and other biological & structural properties, the 47 different serotypes human adenovirus are subdivided into six groups of A-F (Wadell, 1987). The most commonly used adenoviruses in laboratory studies with cultured cells are the human types 2 and 5. They belong to non-oncogenic group C.

1.1. Architecture of an adenovirion

Electron micrographs show that the adenoviruses all have a very characteristic icosahedral structure about 80nm in diameter (Tooze, 1973). The virion of an adenovirus consists

of an outer capsid (a protein shell) and a dense central core (viral genome associated with virion polypeptides).

Capsid The capsid of an adenovirion comprises 252 capsomers that are arranged into an icosahedron about 80nm in diameter. Among these, the 240 capsomers that occupy the facets and edges of the icosahedron are called hexons (they have six neighbors), and the remaining 12 are pentons (they have five neighbors instead). The penton is made up with a penton base that sits at the vertice of the icosahedron and a fiber that projects from the base. The hexons are either "peripentonal' or in the "group of nine" distinguished by their location in the capsid (Fig. 1. A.). Peripentonal hexons are the hexons that surround the pentons, and all the rest of the hexons are included in the groups of nine hexons since these nine hexons are released as a whole after virion disruption (Tooze, 1973).

Core The major components of a core of an adenovirion comprise one linear double-stranded DNA molecule of approximate 35 kilobase pairs long, two proteins that closely associate with the DNA to form a DNA-protein complex, and two copies of a 55K terminal protein covalently linked to the 5' ends of the DNA (Russell et al, 1971; Rekosh et al, 1977). The DNA-protein complex is formed in such a manner that the DNA molecule can be compacted over 5-fold in length in the core (Mirza and Weber, 1982).

Virion polypeptides SDS polyacrylamide gel electrophoresis analysis show that there are at least eleven polypeptides in an adenovirion (Fig. 1. B.). Polypeptides II (120K), III (85K) and IV (62K) present three major capsid proteins of hexon,

penton base and fiber respectively: The hexon is a trimer of three identical polypeptides II held tightly together by non-covalent interactions, the penton base is a pentamer of

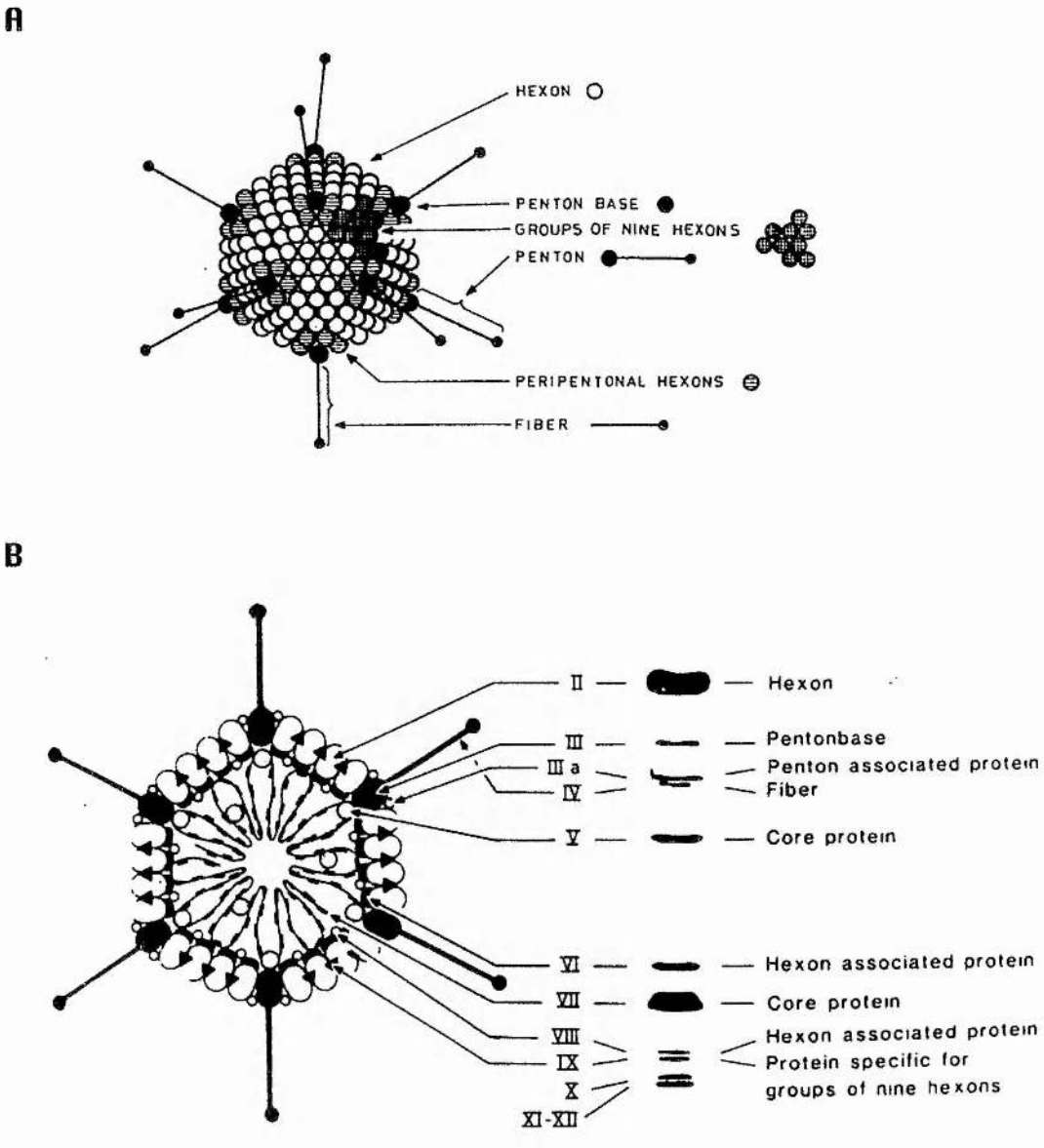


Fig. 1. Schematic representation of an adenovirion
A. The location of various components in the adenovirion capsid (from Tooz, 1973 pp.424)
B. The polypeptides of the adenovirion resolved by SDS-polyacrylamide gel electrophoresis (from Horwitz, 1986 pp.565)

polypeptide III and the fiber is a trimer of polypeptide IV. The trimer of polypeptide IV non-covalently attaches to the pentamer of polypeptide III to form the penton. The hexons, penton bases and fibers of the capsid are the proteins that carry various antigenic determinants of the adenoviruses. Thus they are very important in viral classification and clinical diagnosis. Minor components of capsid proteins are polypeptides VI (24K), VIII (13K), IX (12K) and IIIa (66K), of which, VI, VIII and IX are found to be associated with the hexons, and IIIa appears to be in contact with both penton base and peripentonal hexon. It may also extend through the capsid and have contact with core proteins, thus IIIa may serve a bridging function between the core and the capsid (Horwitz, 1986). Polypeptides V (48.5K) and VII (18.5K) are two major proteins in the core. They are both rich in arginine and other basic amino acids, and they are both non-covalently linked to the DNA to form the complex. But polypeptide VII binds to DNA more tightly than polypeptide V. The complex between polypeptide VII and DNA is very stable. On mild dissociation with pyridine or sodium deoxycholate, the latter can be easily removed from the complex while the former can not. The amount of polypeptide VII in the core is also more abundant than polypeptide V (about 6-fold).

1.2. Genome organisation

The adenovirus genome is a linear double-stranded DNA molecule of approximate 35 kilobase pairs (Kbp) in length. Each strand has a short sequence of about 100bp long at one end that is complementary to the sequence at the other end,

i.e. an inverted terminal repeat (ITR) structure, and a 55K terminal protein (TP) covalently linked to the 5' end. The whole genome is conventionally divided into 0-100 map units (m.u) from the extreme left-hand end to the extreme right (Fig.2; Horwitz, 1986). There are four early and five late regions or transcription units that have been mapped in the genome.

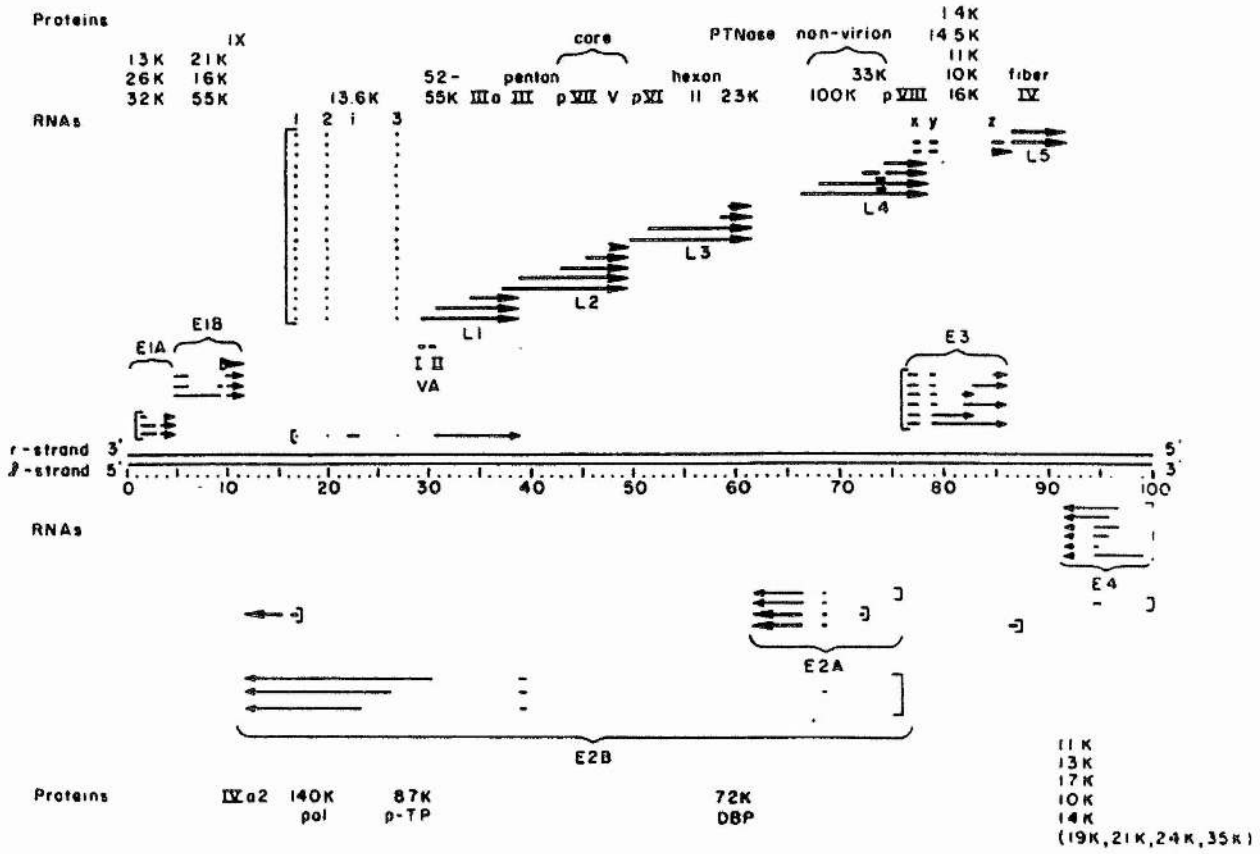


Fig. 2. The adenovirus genome and its RNA transcripts. The proteins encoded by each RNA group are listed either above or below this group. The roman numerals indicate virion polypeptides and nonstructural proteins are indicated in kilodaltons, some by function as well (from Horwitz, M. S. 1986 pp.588).

From left to right, the right (r) strand which is transcribed rightwards contains early region 1 (E1; m.u 1.3-11.2), late regions 1 to 5 (L1-L5; m.u 16.45-99), and early region 3 (E3; m.u 76.6-86.2). From right to left, the left (l) strand, transcribed to the left, contains early regions 4 (E4; m.u 96.8-91.3) and 2 (E2, m.u 75.2-14.2). The "early" (E) designates those regions of the genome which are expressed before the onset of viral DNA replication. Once viral DNA synthesis is initiated, the gene expression turns to"late" (L).

Early regions 1 and 2 (E1 & E2) E1 is subdivided into E1A (m.u 1.3-4.5) and E1B (m.u 4.6-11.2). Because it specifies two separate transcription units by mapping of promoter sites and mRNAs within this site, there are several regulatory elements of E1A. Three major mRNAs of 13S, 12S and 9S are generated from this promoter by different splicing. Of these, 13S mRNA codes for a 289 amino acid residues long (289R) protein that is required for the activation of all other early gene expression and 12S mRNA codes for a 243R protein. The E1B promoter site is located at m.u 4.7. Similarly it generates two major mRNAs of 22S and 13S, coding for a 55K and 19K polypeptides. Both E1A and E1B proteins are tumour antigens (reviewed by Boulanger and Blair, 1991). The E2 region is also subdivided into E2A (m.u 67.9-61.5) and E2B (m.u 29-14.2). They share the same promoter which is located at m.u 75 during early times of infection and shifted to m.u 72 at late times as viral DNA synthesis has been initiated. E2A codes for a 72K multifunctional DNA-binding protein (DBP) and E2B codes for a 87K preterminal protein (pTP) and a 140K DNA polymerase

(pol). DBP, pTP and pol are three viral replication proteins (Akusjarvi et al, 1986).

Late regions 1 to 5 (L1-L5) Late transcription units are characterized by polyadenylation sites of their transcripts. There are five possible locations of polyadenylation sites that lie downstream along the genome, generating five families of L1 to L5 (Fig. 2). The mRNAs in each family have common 5' and 3' termini, and differ by different length of intron being removed during splicing. All late mRNAs are generated from a single promoter—the major late promoter (MLP) at m.u 16.45. They encode mainly viral structural proteins as indicated in Fig. 2.

1.3. Multiplication cycle

Comparing with other viruses, adenoviruses have a prolonged period of about 12-18 hours for multiplication. This differs with multiplicity of infection, cell-virus system and physiological conditions.

Attachment, penetration and uncoating It has been suggested (Philipson et al, 1968) that an adenovirion attaches to a cell by binding to its specific receptors on the membrane of the cell. There are approximately 10^4 virion receptors and 10^5 fiber receptors of adenoviruses in each host cell membrane. This implies that the binding mechanism is multivalent. An early study (Morgan et al, 1969) showed that the particles of adenoviruses enter the cell by passing directly through the plasma membrane, i.e. translocation. But there is increasing evidence showing that the entry could also be receptor-mediated endocytosis (Chardonnet and Dales,

1970; Svensson, 1985). As adenoviruses enter the cell, it either loses some pentons and peripentonal hexons (Sussenbach, 1967), resulting in partial uncoating of the virion; or leads to a conformational change of the capsid in the endosome vesicles, resulting in loss of capsid proteins, e.g. a conformation change in hexon induced by low pH results in the exposure of endoproteolytic sites of the protein (Everitt et al, 1988). Once the capsid is removed, the core migrates to the cell nucleus. There viral DNA is released and transcription takes place.

Transcription The adenovirus genome is transcribed in sequences (i.e. transcription units) by its host cell DNA-dependent RNA polymerase II. There are two stages of transcription – early and late in an adenovirus infection. Viral regulatory and replication proteins are made from mRNAs transcribed at the early stage and virion proteins are made at the late stage. Early transcription starts from the E1A region immediately after infection. E1A 289R protein possesses a transactivation activity and stimulates the transcription directed by RNA polymerase II from many promoters including E1B, E2, E3, E4 and E1A itself. This is accomplished by altering the activity of various transcription factors that recognize the specific regulatory sequences upstream from the promoters (Flint and Shenk, 1989). Thus transcription of the E1A region is followed by transcription of regions E1B, E3, E4, and last of all, region E2. In addition to E1A 289R protein, E2 early transcription also requires an E4 gene product, a 17K polypeptide (Babiss, 1989; Reichel et al, 1989). The E2 region is of some special interest. It is the only transcription unit in an

adenovirus that appears to use alternative promoter sites for its transcription. At early times, the early promoter of E2 is activated by a cellular DNA-binding factor E2F while the activation of E2F requires E1A 289R and E4 17K proteins. In contrast, the E2 late promoter, which is active only at late phase of infection, is refractory to the transactivation of E1A 289R protein (Berk, 1986). Its activition undergoes a different mechanism which is currently unclear. Both early and late promoters of E2 generate two groups of spliced primary transcripts E2A mRNA and E2B mRNA. The amount of the two mRNAs differs greatly one from the other. E2B mRNA is about 100-fold lesser than E2A mRNA (Stillman et al, 1981). Alternative and independent function of two promoters allows transcription of E2 region to be regulated independently at early and late times. Once viral DNA replication begins, late gene transcription becomes dominant. The late mRNAs and the proteins they encode are produced in large quantities at late times (Flint, 1982).

DNA replication There are two characteristics in the adenovirus DNA replication: it always begins directly at either end of the DNA, continuously but asynchronously, in a 5' to 3' direction; it does not require RNA primers, instead it employs a protein-priming mechanism in which a precursor of the viral terminal protein (pTP) is covalently bound to a dCMP residue of the viral terminal nucleotide, i.e. the 3'-OH group in the pTP-dCMP complex is used as a primer. This form of DNA synthesis involves a displacement process. When DNA synthesis starts, only one strand serves as a template. As the synthesis proceeds, the non-template strand is gradually

released from the parental DNA molecule and finally displaced by the newly synthesized DNA. The displaced single strand can then form a panhandle structure resulting from base pairing of its inverted terminal repeats (ITRs) and serve as a template for a second round of DNA synthesis (reviewed by Hay and Russell, 1989; Challberg and Kelly, 1989; Stillman, 1989).

Extensive mutational analysis has defined that the origin of adenovirus DNA replication is located within the terminal 51bp of the genome at either end. It comprises a highly conserved core domain (terminal 1-18 bp) which alone is able to support initiation of DNA replication both in vivo and in vitro but only to a limited extent (Challberg and Rawlins, 1984; Hay, 1985; Wides et al, 1987), and an auxiliary region (terminal 19-51 bp) which can enhance the initiation reaction up to 100-fold (Rawlins et al, 1984; Rosenfeld et al, 1987). It is now clear that the auxiliary region of the origin contains two cis-acting sequences that are recognized by NF I (cell nuclear factor I) and NF III (cell nuclear factor III), the two cellular sequence-specific DNA-binding proteins that are required for efficient viral DNA replication. One of the mechanisms involved in eukaryote's control of transcription and replication results from the interplay between regulatory DNA sequences and their multiple site-specific DNA- binding proteins (Johnson and Mcknight, 1989). In eukaryatic DNA viruses, the origins of replication frequently contain transcription regulatory sequences that function as auxiliary regions enhancing the initiation of DNA replication (de Pamphilis, 1988). The enhancement is achieved by binding of

this region to its specific protein such as NFI and NF III. NFI and NF III are identical to CCAAT-binding transcription factor CTF and octamer-binding transcription factor OTF-I or Oct-I respectively (Jones et al, 1987; O'Neill et al, 1988). The recognition sequence for NFI is next to the core origin but separated from the core by a precisely defined spacer region, where sequence changes are tolerated while insertions and deletions are not (Adhya et al, 1986; Coenjaerts et al, 1991). NFI binds to DNA as a dimer symmetrically at one side of the helix, making contacts with the major groove of DNA (de Vries et al, 1987; Gounari et al, 1990). The recognition sequence for NF III is immediatly adjacent to the NFI's and actually overlap for two nucleotides. Insertion of two or more base pairs between the two sequences almost completely abolishes the stimulation of initiation raised by NF III (Pruijn et al, 1988; Coenjaerts et al, 1991), suggesting that the position of NF III binding site is essential for its enhancement. NF III binds to DNA at both sides of the helix, making contacts with both the major and minor grooves of DNA. Although the two proteins are in close proximity, no contacts between them have been demonstrated so far. They function independently, employing different mechanisms (Mul et al, 1990). NFI is fully capable of stimulating DNA replication in the absence of NF III and vice versa. However the level of stimulation by NFI is dependent on the concentration of pTP-pol complex, the two closely related viral proteins that are attributed to the centre of DNA replication, and there is an evidence showing that NFI directly interacts with pol (Bosher et al, 1990).

Initiation of adenovirus type 4 (Ad4) DNA replication is an exception to the above description. The inverted terminal repeat of Ad4 does not contain the NFI recognition site. Although it does have a binding site for NF III, neither factor is required for DNA replication in vivo or is capable of stimulating DNA replication in vitro (Hay et al, 1988). It has been demonstrated (Hay, 1985; Harris and Hay, 1988) that an efficient DNA replication of Ad4 requires only the terminal 18bp of the genome, i.e. the core origin, and the three viral proteins: pTP (preterminal protein), pol (DNA polymerase) and DBP (DNA-binding protein). This implies that the terminal 18 bp of the genome constitutes a fully functional origin of DNA replication and it can be recognized by viral proteins in the absence of cellular proteins. Gel electrophoresis DNA-binding and DNase I footprint analysis have demonstrated that the heterodimer of pTP-pol complex specifically recognizes the sequence of viral origin within terminal 1-18 bp and protects its 8-17 bp from nuclease cleavage (Temperley and Hay, 1992).

The initiation takes place when a covalent bond between the α-phosphoryl group of either terminal dCMP residue in DNA and the β-OH group of a serine residue in pTP is formed. Five proteins are involved in this reaction as mentioned above. However pTP and pol alone either cannot bind to the DNA origin (Chen et al, 1990) or have relatively low specificity and affinity for the origin (Temperley and Hay, 1992). They must function as a complex. The complex of pTP-pol binds to DNA preferably to single-stranded (Kenny and Hurwitz, 1988; Chen et al, 1990), suggesting that an unwound region of origin

must be presented prior to the binding. NFI and NF III can bind to their recognition sites independently, and the binding of NFI to DNA is enhanced by DBP (Stuiver and van der Vliet, 1990). DBP is also able to stimulate initiation in the absence of NFI with a currently unknown mechanism. NFI interacts with pol as well and in doing so directs pTP-pol to the origin, and at the same time stabilizes the complex between pTP-pol and core origin (Mul and van der Vliet, 1992). Thus it can be concluded from these results that protein-protein and protein-DNA interactions at the origin are indeed required prior to initiation, leading to the formation of an efficient preinitiation complex containing all the five proteins and the origin or only the three viral proteins and the origin, and the generation of a functional partially single-stranded DNA template.

Once the initiation has taken place, further DNA synthesis falls into the strand displacement mechanism cooperated by pol and DBP. Complete chain elongation of nascent DNA molecule requires an additional cellular protein NF II (cell nuclear factor II) which has a topoisomerase I activity to remove torsional strain induced by the displacement process (Nagata et al, 1983).

Assembly and release Virion assembly is confined to the nucleus, and it begins when single polypeptides are assembled into capsomers (Russell et al, 1967; Horwitz et al, 1969). The formation of hexon capsomers from monomers (polypeptide II) to a trimer is mediated by a virus-coded 100K protein (Cepko and Sharp, 1983). It is a nonstructural late protein and present in large quantities in infected cell

extracts but absent from mature virions. When hexon capsomers are formed, they then self-assemble into a empty procapsid, presumably through the formation of ninemers with polypeptide that would occupy each of the 20 faces of the virion. Proteins that associate with hexon ninemers at this stage are precursors of polypeptides VI (pVI) and VIII (pVIII). The formation of penton capsomers from five penton base and three fiber polypeptides (III and IV respectively) is not clear. Following procapsid formation, the DNA and core proteins are inserted. It appears that the insertion of DNA is progressed in the absence of core proteins, thus another basic protein would be needed to neutralize the charge of the DNA before its packaging. This basic protein possibly is the virus-coded DBP (Nicolas et al, 1983). Because the left first 400 bp of the gemome contains a specific recognition sequence for DNA packaging (Hammarskjold and Winberg, 1980), the DNA enters the preformed capsid always preferentially from the left-hand end. Following insertion of DNA and core proteins, the proteolytic process of precursors (e.g. pVI, pVII, pVIII and pTP) is carried out by a virus-coded 23K protease (Webster et al, 1989). The virion particle then tightens its configuration and becomes mature. Virions assembly can be blocked by arginine deprivation (Russell and Becker, 1968). Assembled virions are accumulated in the nucleus, where they form crystalline aggregates which can be seen by electron microscopy. They are released from the nucleus only when the nuclear membrane is disrupted, and then escape from the host cell by cell lysis.

2. Protein phosphorylation

Phosphorylation is one of the most common types of protein posttranslational modification that is now recognized to mediate the response of eukaryotic cells to external stimuli (Krebs, 1986). The reaction of phosphorylation is catalyzed by protein kinases the enzymes that transfer phosphates from phosphoryl donors to acceptor amino acid residues of protein substrates. It is reversible. The reverse reaction, i.e. dephosphorylation is catalyzed by phosphoprotein phosphatases (Ballou and Fischer, 1986), the enzymes that take the phosphates off from the acceptor residues of protein substrates. Phosphorylation/ dephosphorylation reveals a general mechanism by which different cellular function are controlled. This has not been realized until the discovery that glycogen metabolism is controlled by phosphorylation (Krebs et al, 1959). Glycogen phosphorylase kinase was the first protein kinase to be purified. Following the discovery of cyclic AMP-dependent protein kinase (Walsh et al, 1968), the number of enzymes that have been identified as protein kinases have risen exponentially (Hunter, 1987). All protein kinases thus far characterized with regard to substrate specificity (i.e. phosphoryl acceptor residues of the protein) fall within one of two broad classes, serine/threonine-specific protein kinases (Edelman et al, 1987) and tyrosine-specific protein kinases (Hunter and Cooper, 1985). Protein phosphorylation occurs most frequently on seryl and threonyl residues and less frequently on tyrosyl residues (Cooper et al, 1983). The discovery of protein tyrosine kinases took place about 20

years later than that of protein serine/threonine kinases (Hunter, 1987). The two protein kinases can be further characterized into several subclasses e.g. protein serine/threonine kinases can be further divided into cyclic nucleotide-dependent subfamily, Raf-Mos proto-oncogene subfamily and casein kinase subfamily etc, and protein tyrosine kinases can be further divided into Src oncogene subfamily, Abl oncogene subfamily and growth factor receptor subfamily etc. However, all eucaryotic protein kinases are evolutionarily related with common properties (Hanks et al, 1988).

2.1. General properties of protein kinases

All protein kinases require divalent metal ions for their activity. Mg^{++} probably serves as the physiologically significant cation in all instances (Krebs, 1986). Many protein tyrosine kinases show an apparent preference for Mn^{++} over Mg^{++} due to indirect inhibition of Mn^{++} to phosphotyrosine-specific phosphatases (Hunter and cooper, 1985), and probably Mn^{++} mimics the action of a physiological activitor (Wente et al, 1990). ATP is the preferred nucleotide of phosphoryl donor for almost all protein kinase since there are ATP recognition sites in the conserved catalytic domain of the kinases (Hanks et al, 1988; Soderling, 1990).

Conserved catalytic domain Protein kinases principally have two functional domains: a catalytic domain and a regulatory domain (Taylor, 1989). The catalytic domain of the kinase lies near the carboxyl terminus of the protein, comprising 250 to 300 amino acids. Of these, some regions are highly conserved

throughout all the protein kinase family, and some have lower conservation. Starting from the N-terminus of the catalytic domain, the following consensus motif of Gly-X-Gly-X_2-Gly-X_{15-20}-Lys is present in all protein kinases and responsible for ATP binding (Hanks et al, 1988). The first two Glys and the Lys are invariant residues, but the third Gly residue is replaced by Ser or Ala in some instances (Soderling, 1990). X_s are nonconserved amino acids. The short sequence Gly-X-Gly-X-X-Gly that is also found in many nucleotide-binding proteins probably serves as a nucleotide fold motif (Rossman et al, 1974; Wierenga and Hol, 1983): It forms an elbow around the nucleotide, with the first glycine in contact with the ribose moiety and the second glycine lying near the terminal pyrophosphate (Sternberg and Taylor, 1984). In two positions downstream of the third Gly, there is a highly conserved Val. This valine residue may contribute to the positioning of the conserved glycines (Hanks et al, 1988). The invariant Lys of the consensus appears to be directly involved in ATP-binding (Taylor, 1989). Since fluorosulfonylbenzoyl 5'-adenosine (FSBA), an analog of ATP, inhibits the function of the catalytic domain by covalently modifying this Lys (Zoller et al, 1981), mutations at this position result in loss of protein kinase activity (Hannink and Conoghue, 1985; Weinmaster et al, 1986; Chen et al, 1987; Glenney et al, 1988). About 80-180 residues (Hunter, 1987) downstream from the Lys, there are a series of highly conserved short stretches and individual amino acid residues separated by regions with lower conservation, forming a central core of the catalytic domain. The following consensus triplets Arg-Asp-Leu, Asp-Phe-Gly and Ala-Pro-Glu

are of characteristic feature of the centre core. They are involved in substrate recognition (Hanks et al, 1988; Taylor, 1989) and enzyme catalysis e.g. the consensus *Arg-Asp-Leu*-Lys-Pro-Glu-Asn specifies serine/threonine kinases, having a requirement for basic aminoacids preceding the phosphorylation site and the consensus *Arg-Asp-Leu*-Arg-Ala-Ala-Asn (src family only) or *Arg-Asp-Leu*-Ala-Ala-Arg-Asn (all others) specifies tyrosine kinases, having a requirement for acidic residues. The consensus triplet Ala-Pro-Glu is often mentioned as a key protein kinase catalytic domain indicator (Hunter and Cooper, 1986). Each individual aminoacid in this triplet is required for enzyme activity. The Asps of the first and second triplets are also involved in ATP-binding (Brenner, 1987; Buechler and Taylor, 1988). In contrast, the regulatory domain of the kinase shows no overall evolutionary relatedness but the mechanism by which the regulatory domain maintains the catalytic domain in an inactive state in the absence of ligands appears to be similar.

Substrate recognition It is common that a single protein can be phosphorylated at several sites by different protein kinases (Cohen, 1985), and a single protein kinase is able to phosphorylate several different substrate proteins (Engstrom, 1984). This is determined partially by the primary structures of both kinases and their substrate proteins. As mentioned before, there are substrate recognition sites in the conserved kinase catalytic domain, which require not only the phosphorylatable target hydroxyamino acid but also the neighbourhood amino acids. The overall similarity of the conserved catalytic domain in the protein kinase family would

result in the similarity of substrate selection in each family. For protein serine/threonine kinases in every case the canonical recognition sequence contains basic residues near the serine or threonine e.g. sequence Arg-Arg-X-Ser-Y, in which X specifies any amino acid and Y is a hydrophobic amino acid, is specifically favoured by cAMP-dependent protein kinase (Zetterqvist et al, 1990). For protein tyrosine kinases, usually a sequence that contains acidic residues proximal to the target tyrosine is always beneficial (Geanlen and Harrison, 1990). However, in addition to primary sequences, there are factors that determine the specificities for individual kinase in substrate recognition. The secondary and/or tertiary structure of the protein is surely an important factor in this process. Biological function requirement of the kinase and substrate protein could be another factor e.g. substrates for viral tyrosine kinases are of two sorts: glycolytic enzymes and cytoskeletal proteins (Hunter and Cooper, 1986). Substrates for cellular tyrosine kinases are mainly three types (Glenney, 1991): membrane and cytoskeletal proteins such as Ezrin (Bretscher, 1989) and Paxillin (Tumer et al, 1990); serine/threonine protein kinases that regulate the cell cycle such as $p34^{cdc2}$ (Gould and Nurse, 1989) and MAP (M phase activating protein) (Anderson et al, 1990a); and SH2-domain containing proteins that transduce signals from growth receptors such as PLCγ (Phospholipase C γ) (Morrison et al, 1990) and GAP (GTPase activating protein) (Kazlauskas et al, 1990).

<u>Autophosphorylation</u> Almost all protein kinases possess an ability to undergo autophosphorylation process, i.e. they

phosphorylate themselves (krebs, 1986). The autophosphorylation reaction can be either intramolecular or intermolecular. But the functional meaning of the process is not very clear in many individual case. In general, autophosphorylation stimulates kinase activity. This is probably because many enzymes are kept in an inactive or inhibited state by the interactions of their regulatory domain and catalytic domain (Taylor, 1989). Autophosphorylation may disrupt the interactions between the two domains by inducing a conformational change in the enzyme, thereby releasing the catalytic domain from the inhibition of the regulatory domain (Soderling, 1990). In cAMP kinase, autophosphorylation causes a 10-fold increase in the dissociation constant of interactions between regulatory subunits and catalytic subunits (Scott and Mumby, 1985), whereas in cGMP kinase, the effect of autophosphorylation on the activation of the enzyme is mediated by a change in the affinity of the kinase for cyclic AMP (Landgraf et al, 1986). The requirement for autophosphorylation of CaM kinase II (Calcium/calmodulin-dependent protein kinase II) at Thr-286 is to generate a partially Ca^{++}-independent species of the kinase and block the inhibitory interactions between the inhibitory domain and the ATP-binding site (Colbran et al, 1989; Fong et al, 1989). Autophosphorylation is particularly important in regulating functions of protein tyrosine kinases, and all protein tyrosine kinases identified so far autophosphorylate at multiple sites, especially receptor type kinases (Hunter and Cooper, 1985; 1986). One major autophosphorylation site which is highly conserved throughout the family is present in the central core

of the catalytic domain (Hanks et al, 1988). Autophosphorylation of this site in $pp60^{c-src}$ (corresponding to Tyr-416) controls its kinase and transforming activity (Piwnica-Worms et al, 1987; Kmiecik and Shalloway, 1987). In the PDGF receptor, autophosphorylation of Tyr-751, which is located within the insert region, regulates interactions of the receptor with cellular substrates (Kazlauskas and Cooper, 1989). In EGF receptor, autophosphorylation appears to facilitate a conformation that is competent to interact with and phosphorylate cellular substrates since autophosphorylation sites of the receptor compete with exogenous substrates for the substrate binding site of the kinase (Honegger et al, 1988).

Oncogene products Oncogenes were originally thought to be retrovirus-encoded genes that induced tumors in birds and rodents, but have now been shown to be derived from normal cellular genes that have been picked up by retrovirus and genetically damaged (Bishop, 1991). They are dominant mutated forms of host genes (colloquially termed proto-oncogenes). Oncogenes encode a large number of proteins that fall into several classes (Table.1.) including growth factors and their receptors, membrane-associated mutant proteins, and cytoplasmic & nuclear oncoproteins (Hunter, 1991). Surprisingly, many cellular protein kinases, particularly tyrosine kinases, are found to be homologous to many of these oncoproteins on the list (Table.1.). They are actually viral kinases or matant forms of cellular kinases encoded by oncogenes that are derived from normal kinase genes (Hunter and Cooper, 1986; Yarden and Ullrich, 1988;

Hunter, 1991). Thus the two kinases (viral and their counterpart cellular) are genetically related homologues. They have the same kinase activity. This implicates the role that phosphorylation especially tyrosine phosphorylation plays in cell transformation.

Table 1. Functions of Cell-Derived Oncogene Products

Class 1—Growth Factors	
sis	PDGF B-chain growth factor
int-2	FGF-related growth factor
hst (KS3)	FGF-related growth factor
FGF-5	FGF-related growth factor
int-1	Growth factor?
Class 2—Receptor and Nonreceptor Protein-Tyrosine Kinases	
src	Membrane-associated nonreceptor protein-tyrosine kinase
yes	Membrane-associated nonreceptor protein-tyrosine kinase
fgr	Membrane-associated nonreceptor protein-tyrosine kinase
lck	Membrane-associated nonreceptor protein-tyrosine kinase
fps/fes	Nonreceptor protein-tyrosine kinase
abl/bcr–abl	Nonreceptor protein-tyrosine kinase
ros	Membrane associated receptor-like protein-tyrosine kinase
erbB	Truncated EGF receptor protein-tyrosine kinase
neu	Receptor-like protein-tyrosine kinase
fms	Mutant CSF-1 receptor protein-tyrosine kinase
met	Soluble truncated receptor-like protein-tyrosine kinase
trk	Soluble truncated receptor-like protein-tyrosine kinase
kit (*W* locus)	Truncated stem cell receptor protein-tyrosine kinase
sea	Membrane-associated truncated receptor-like protein-tyrosine kinase
ret	Truncated receptor-like protein-tyrosine kinase
Class 3—Receptors Lacking Protein Kinase Activity	
mas	Angiotensin receptor
Class 4—Membrane-Associated G Proteins	
H-*ras*	Membrane-associated GTP-binding/GTPase
K-*ras*	Membrane-associated GTP binding/GTPase
N-*ras*	Membrane-associated GTP-binding/GTPase

Table 1. *Continued.*

gsp	Mutant activated form of G_s α
gip	Mutant activated form of G_i α
Class 5—Cytoplasmic Protein-Serine Kinases	
raf/mil	Cytoplasmic protein-serine kinase
pim-1	Cytoplasmic protein-serine kinase
mos	Cytoplasmic protein-serine kinase (cytostatic factor)
cot	Cytoplasmic protein-serine kinase?
Class 6—Cytoplasmic Regulators	
crk	SH-2/3 protein that binds to (and regulates?) phosphotyrosine-containing proteins
Class 7—Nuclear Transcription Factors	
myc	Sequence-specific DNA-binding protein
N-*myc*	Sequence-specific DNA-binding protein?
L-*myc*	Sequence-specific DNA-binding protein?
myb	Sequence-specific DNA-binding protein
lyl-1	Sequence-specific DNA-binding protein?
p53	Mutant form may sequester wild-type p53 growth suppressor
fos	Combines with c-*jun* product to form AP-1 transcription factor
jun	Sequence-specific DNA-binding protein; part of AP-1
erbA	Dominant negative mutant thyroxine (T_3) receptor
rel	Dominant negative mutant NF-κB-related protein
vav	Transcription factor?
ets	Sequence-specific DNA-binding protein
ski	Transcription factor?
evi-1	Transcription factor?
gli-1	Transcription factor?
maf	Transcription factor?
pbx	Chimeric E2A–homeobox transcripton factor
Hox2.4	Transcription factor?
Unclassified	
dbl	Cytoplasmic truncated cytoskeletal protein?
bcl-2	Plasma membrane signal transducer?

Table.1. Functions of cell-derived oncogene products (from Hunter, 1991).

2.2. Functions of protein phosphorylation

Protein phosphorylation is now recognized as a universal control mechanism that regulates eukaryotic cell functions. It involves the control of signalling pathways that allow the cell to respond to external stimuli and is involved in the control of intramolecular biochemical changes that are required for cell growth and division. Because many oncogene products are mutant forms of protein tyrosine kinases, tyrosine phosphorylation is closely related to cell transformation.

Control of cell cycle A cell cycle is defined as a period during which a series of important events required for successful cell reproduction are completed. Two hallmarks of cellular reproduction are chromosomes replication in S-phase (Laskey et al, 1989) and mitosis (the replicated chromosomes are segregated into two daughter cells) in M-phase (Mcintosh and Koonce, 1989). Thus entry to S-phase and M-phase are two restriction points that govern the cell cycle. It is now clear that the entry to M-phase is controlled by a key regulator originally named maturation promoting factor (MPF) but now considered as M phase promoting factor or M phase kinase, since activated MPF phosphorylates histone H1 and other important proteins, leading to the major events of M-phase (Murray and Kirschner, k1989; Doree, 1990). MPF consists of two distinct components: a catalytic subunit of a 34K protein kinase from cdc2 ($p34^{cdc2}$) and a regulatory subunit called cyclin because of its cyclic appearance (Lewin, 1990). $p34^{cdc2}$ is a homologous product of cell division cycle gene 2 (cdc2) from fission yeast Schizosaccharomyces pombe (S pombe) but conserved in all eukaryotic cells (Pines and Hunter, 1990). It is

a protein serine/threonine kinase and its activity is itself regulated by phosphorylation and dephosphorylation on tyrosine and threonine residues, i.e. being active when dephosphorylated and inactive when phosphorylated (Draetta et al, 1988; Moria et al, 1989). Activation of $p34^{cdc2}$ (i.e. being dephosphorylated) induces M-phase whereas exit from M-phase requires inactivation of $p34^{cdc2}$ (i.e. being phosphorylated again) (Nurse, 1990).

Signal transduction A common mechanism by which external stimuli such as growth factors, differentiation factors and hormones regulate cellular proliferaton and differentiation is through transmembrane receptors with inducible protein-tyrosine kinase activity (Ullrich and Schlessinger, 1990). Binding of these receptors to their specific ligands results in a conformational change in the extracellular domain of the receptors, which in turn transmit across the membrane to their cytoplasmic domain that contains a tyrosine kinase catalytic domain by oligomerization (Schlessinger, 1988) or dimerization (Seifert et al, 1989; Heldin et al, 1989). Cross-phosphorylation then occurs intramolecularly or intermolecularly on multiple tyrosine residues, allowing further change of conformation that enhances kinase activity toward other substrates and provides binding sites for SH2 domain containing proteins (Cantley et al, 1991). The SH2 domain (Koch et al, 1991)-containing proteins are a series of cytoplasmic signalling molecules that all contain a region named src homology region 2 (SH2). Since this region is homologous to a conserved noncatalytic region of src tyrosine kinase family (Sacowski et al, 1986; Pawson, 1988), the SH2

domain binds to a tyrosine-phosphorylated protein (Anderson et al, 1990; Mayer et al,1991), and different SH2-containing molecules would recognize different phosphotyrosine sites (Fantl et al, 1992). The binding of receptors to SH2 domain-containing proteins results in the formation of heteromeric protein complexes at or near the plasma membrane, which would initiate further response to the external stimuli along the signal transduction pathways (Cantley et al, 1991). The nonreceptor family of Src tyrosine kinases mediate signal transduction via the SH2 domain by phosphorylating the target protein first (Cantley et al, 1991). Thus it is likely that tyrosine phosphorylation acts as a switch that controls the activation of signal transduction pathways.

Cell transformation Tyrosine-specific protein kinase activity was first discovered in cells transformd by Rous Sarcoma Virus (Collett and Erikson, 1978; Hunter and Sefton, 1980). Since then many protein tyrosine kinases have been discovered as oncogene products (Hunter, 1991) and their importance in regulating cell functions has been revealed only through the study of oncogene and protooncogenes (Bishop, 1991). So it is not surprising that tyrosine phosphorylation is deeply involved in cell transformation. Both oncogenes and their counterpart proto-oncogenes send their signals through the same pathways in a similar manner (Cantley et al). When the oncogene becomes dominant, the normal negative regulatory control of cellular tyrosine kinases appears to be abrogated. Oncogenes derived from receptor tyrosine kinases such as v-erbB (from EGFR) and v-kit (from PDGFR), or encode mutant proteins that associate with the inner face of the

cytoplasmic membrane such as v-Src and v-Ras, provide a continuous ligand-independent mitogenic signal (Hunter, 1991); Oncogenes that encode mutant forms of cytoplasmic protein serine/threonine kinases transform cells by acting on check points of the cell cycle (Hunter, 1991) e.g. c-Mos (coding for a protein serine kinase) and stabilizes the activity of MPF and is involved in the mechanisms by which cells are prevented from entering mitosis when they are too small or when DNA synthesis has not been completed (Sagata et al, 1989a; 1989b). In contrast v-Mos drives cells into mitosis by accelerating the activation of the cyclin-cdc2 complex.

3. Adenovirus DNA-binding protein (DBP)

The adenovirus DNA-binding protein (DBP) is a gene product of early region E2A. It is a multifunctional phosphoprotein which is produced in large quantities and accumulates in the nuclei of infected cells at both early and late times (Axelrod, 1978; Sugawara et al, 1977; Doelkerding and Klessig, 1986). DBP contains 529 amino acid of known sequence but has an apparent molecular weight of 72K upon SDS-polyacrylamide gel electrophoresis (Kruijer et al, 1982). Genetic and mutation analyses have shown that DBP contains two functional domains (Rice and Klessig, 1984) which can be separated by mild chymotrypsin treatment (Klein et al, 1979; Linne and Philipson, 1980). The N-terminal domain, consisting of about one-third of the molecule (residues 1-173), is poorly conserved but highly phosphorylated. It does not bind to DNA. Mutations in this part of the protein affect the host range of

the virus and viral late gene expression (Klessig and Grodzicker, 1979; Anderson et al, 1983; Brough et al, 1985). The N-terminal domain is also very rich in proline residues and contains a high content of both basic and acidic amino acids. Two short stretches of basic amino acids 42-46 and 84-89 have been defined to be involved in nuclear transport of DBP (Morin et al, 1989a). In contrast, the C-terminal domain, consisting of two-third of the molecule (residues 174-529), is poorly phosphorylated but highly conserved (Kitchingman, 1985; Vos et al, 1988). DNA and RNA binding activity of the protein is confined to this domain (Klein et al, 1979; Cleghon and Klessig, 1986). The roles that DBP plays in viral DNA replication also appear to be confined to this domain (Friefeld et al, 1983; Tsernoglou, 1985). Mutations in this part of the protein display a DNA replication deficient phenotype (Kruijer et al, 1983).

3.1. Phosphorylation of DBP

DBP is phosphorylated during the course of infection since in vivo labelling of Ad5 infected HEK cells with ^{32}P resolves a phosphorylated polypeptide of apparent molecular weight 72K after SDS PAGE analysis (Russell and Blair, 1977). Most phosphorylation sites of DBP are located at the N-terminal. This is demonstrated by labelling of adenovirus infected cells with both ^{35}S and ^{32}P *in vivo* followed by chymotrypsin cleavage (Klein et al, 1979; Linne and Philipson, 1980) and by amino acids mapping (Anderson et al, 1985). All phosphorylation sites found in DBP are on the serine and threonine residues (Linne et al, 1977; Axelrod, 1978). However

one tyrosine at C-terminal is found to be phosphorylated early after infection (Russell et al, 1989). Bacterial alkaline phosphatase treatment of DBP removes 90-95% phosphate groups from phosphoserines and phosphothreonines (Klein et al, 1979), and reduces apparent molecular weight of DBP from 72K to 70K. This suggests that most of the phosphate associated with DBP is sensitive to alkaline phosphatase and the increase of the 2K in apparent molecular weight of DBP is due to posttranslational modification of phosphorylation (Linne and Philipson, 1980). The bond between the phosphate moiety and the protein is shown to be alkali-labile (Axelrod, 1978), suggesting a monoester linkage of the phosphate to hydroxyl groups of serine and threonine residues (Linne et al, 1977). DBP can be phosphorylated *in vitro* by a major chromatin-associated protein kinase from uninfected Hela cells (Klein et al, 1979), which phosphorylates H-1, H-2a, and H-4 histones (Schlepper and Knipprs, 1975), and is now known as a protein serine/threonine kinase $p34^{cdc2}$ (Draetta et al, 1988; Arion et al, 1988). It has been proposed that the various phosphorylated forms of DBP may carry out the different roles of this multifunctional protein. But only the effect of this posttranslational modification on DNA binding has been directly tested (Linne and Philipson, 1980; Klein et al, 1979). Some results suggest that phosphorylated and dephosphorylated DBP bind to single-stranded DNA with the same efficiency (Linne and Philipson, 1980), while some results suggest that dephosphorylated DBP binds to DNA more tightly than phosphorylated DBP does (Klein et al, 1979). However, mutations that substitute more than eight

phosphorylation sites of DBP alter its ability to enhance its own synthesis whereas substituting one or a few of these sites has little effect on it (Morin et al, 1989b), suggesting that the overall phosphorylation state of the protein may be important for enhancing its own expression.

3.2. Nucleic acid binding activity

Adenovirus DBP was first isolated by its strong binding to single-stranded DNA (van der Vliet & Levine, 1973) and thus classified as a single-stranded DNA (ssDNA) binding protein (Chase & Williams, 1986). But it also binds to double-stranded DNA (dsDNA) as well as RNA (Fowlkes et al, 1979; Cleghon and Klessig, 1986). The binding of DBP to ssDNA is cooperative with no apparent sequence specificity (Schechter et al, 1980). ssDNA covered with DBP has a regular and locally extended configuration, inducing a considerable tilt of the bases (van Amerongen et al, 1987). The size of binding sites is 10-15 nucleotides from circular dichroism spectroscopy (Kuil et al, 1989) and 16 nucleotides from a spectrofluorometric study (Meyers et al, 1990). In contrast the binding of DBP to dsDNA is noncooperative with similar affinity as to ssDNA, forming a multimeric complex (Stuiver and van der Vliet, 1990). Double stranded DNA complexed with DBP has a rigid structure and is unable to fold back on itself (Stuiver et al, 1992). DBP removes tertiary structures from dsDNA and introduces changes in base-to-base positions in dsDNA. The characteristic of the complex is that DBP rapidly dissociates from dsDNA. The complete dissociation occurs within one minute at room temperature (Stuiver et al, 1992). In contrast, dissociation of

DBP from ssDNA is much slower, indicating a different interaction between DBP-ssDNA and DBP-dsDNA. DBP binds to RNA in a similar cooperative manner as to ssDNA (Cleghon and Klessig, 1986). A variety of approaches have been used to localize the nucleic acid binding region. Limited chymotrypsin digestion generate two distinct fragments and the nucleotides binding activity of DBP is confined to its C-terminal domain which contains four conserved regions (CR) defined from sequence comparative analysis as CR1 (amino acids176-186), CR2 (amino acids 323-330), CR3 (amino acids 464-475) and a zinc finger domain (amino acids 273-286) (Kitchingman, 1985; Vos et al, 1988). Mutagenesis studies on these regions suggests that CR3 and to a lesser extent CR2 are involved in the DNA-binding (Neale and Kitchingman, 1989;1990). A zinc finger (Berg, 1990; Vallee, 1991) binding study of DBP suggests that this motif is required for DNA-binding (Vos et al, 1988a) and the conserved region (amino acids 273-286) that mediates zinc binding is fundamental for DNA-binding (Eagle and Klessig, 1992). Study from photochemical cross-linking suggests that methionine 299 and phenlalanine 418 are probably two contact points (tyrosine 455 could be a third possible site) between DBP and DNA (Cleghon and Klessig, 1992). Deletion of the C-terminal 46 amino acids abolishes DNA binding. It can be concluded from these results, that the precise DNA-binding sites in DBP are still a question, although they appear to be within or near the conserved regions in the C-terminal of the protein.

3.3. The roles in DNA replication

DBP is one of the three viral proteins required for DNA replication in both initiation and elongation. DBP stimulates initiation reaction via NF1 and cooperates with pol to elongate the initiated DNA chain.

Initiation Initiation of DNA replication in prokaryotes (Kornbrg, 1988) as well as in eukaryotes (Hay and Russell, 1989; Challberg and Kelly, 1989) requires a preinitiation complex that is formed and stabilized by protein-DNA and protein-protein interactions at the origin of DNA replication. The formation of such a complex in adenovirus type 2 DNA replication requires the binding of nuclear factor I (NFI) to its recognition sites at the origin (Chen et al, 1990). NFI is presumed to promote initiation by its binding to DNA and its interactions with pol at the origin (Bosher et al, 1990). The role DBP plays here is that it increases the affinity of NFI for its binding sites at the origin and it also increases the rate of association and decreases the rate of dissociation of NFI with the DNA template (Cleat and Hay, 1989). The enhancement of NFI-binding by DBP is probably through the interactions of DBP with DNA rather than NFI since no direct contact between DBP and NFI has been found and preincubation of DNA with DBP results in a similar effect (Stuiver and man der Vliet, 1990). DBP forms a multimeric complex with double-stranded DNA in an apparently noncooperative fashion, thus inducing structural changes in DNA and facilitating the binding of NFI as well as the initiation of DNA replication via NFI (Stuiver et al, 1992). However, in the absence of NFI, DBP itself can still

stimulate the frequency of the initiation reaction (Temperly and Hay, 1991).

Elongation DBP has two functions in elongation. One is to coat the displaced single-stranded DNA as a single-stranded DNA-binding protein, thereby most likely protecting it from nuclease digestion (van der Vliet et al, 1978). The other is to increase the processivity of pol as a specific cooperater in DNA chain synthesis (Lindenbaum et al, 1986). In the first role, DBP could be replaced by other single-stranded DNA-binding proteins, but in the second role it absolutely requires the presence of adenovirus DBP. After initiation, pol dissociates from pTP and associates with DBP, forming a complex (Lindenbaum et al, 1986). In this complex, DBP stabilizes pol and facilitates pol translocating through the duplex DNA template since Ad pol is incapable of translocation through regions of secondary structure.

3.4. The roles in transcription

The functions of DBP in gene transcription are not as clear as in DNA replication. However, in general, DBP has a negative effect on early gene transcription and a positive effect on late gene transcription.

Early gene transcription DBP is responsible for the accumulation of early mRNAs by decreasing the half life of some early mRNAs (Babich and Nevins, 1981) and by inhibiting transcription from the E4 promoter (Handa et al, 1983). A temperature-sensitive mutant of adenovirus type 5 (H5ts125), which is restricted to the early phase of infection when grown at the nonpermissive temperature (Ensinger and

Ginsberg, 1972) and maps to the E2A that codes for DBP, overproduces early viral mRNAs at the nonpermissive temperature (Carter and Blanton, 1978), suggesting a loss of function of DBP that controls the abundance of the early mRNAs of the virus. Pulse-labelling during H5 ts125 infection shows that E4 transcription is repressed at the permissive temperature but not at the nonpermissive temperature (Nevins and Winkler, 1980), suggesting a repression function of DBP to the transcription from E4 region. Furthermore, the repression of E4 transcription results from a block of initiation rather than an inducement of premature termination of transcription, i.e. DBP acts directly on the E4 promoter. Preincubation of viral DNA fragments with purified DBP specifically inhibits the transcription from the E4 promoter, while addition of single-stranded DNA significantly reduces the inhibition of DBP for the E4 promoter (Handa et al, 1983), implying a specific effect of DBP on E4 transcription. In contrast, DBP stimulates the transcription from E1A and E2A promoters (Chang and Shenk, 1990; Morin et al, 1989b).

Late gene transcription DBP stimulates the transcription directed by the major late promoter (MLP) and the stimulation of DBP to MLP is about three-fold greater than that of E1A transactivator protein (Chang and Shenk, 1990). A mutation in the E2A gene encoding the DBP (Klessig and Grodzicker, 1979) enhances the normally depressed level of adenovirus late transcription in monkey cells (Johnston et al, 1985). These results suggest that DBP plays a central role in late gene transcription.

4. Aims of this work

Adenoviruses serve as model systems for studying virus-cell relationships and eukaryotic regulation since they are very easy to maintain in standard tissue culture. The adenovirus encodes a nonstructural multifunctional DNA-binding protein (DBP) which is produced in large quantities at both early and late times of infection. It is evident that DBP plays many important roles during virus multiplication and it has been known for some time that DBP is heavily phosphorylated during infection. Previous experiments also show the presence of a phosphotyrosine residue in DBP (Russell et al, 1989). Protein phosphorylation is believed to be a key mechanism in regulating various functions or activities of a protein. Protein tyrosine phosphorylation especially has been known to have a close relation to viral transformation and cell proliferation. However no specific functions of DBP have been found so far to be attributed to its phosphorylation state. Particularly it has not been confirmed that DBP is phosphorylated at a tyrosine residue. Thus the purpose of this work is to investigate phosphorylation events, particularly tyrosine phosphorylation of DBP in an in vitro system and its relation to various functions of DBP by :

(a) Preparing antibodies against phosphotyrosine and DBP to detect phosphotyrosine in DBP and to characterise the phosphorylation state of DBP.

(b) Constructing an expression vector to carry out site directed mutagenesis on DBP in order to investigate various functions that are ascribed to DBP at the molecular level.

Part I Preparing antibodies against phosphotyrosine and DBP

Monoclonal antibody (MAb) techniques have been well established and developed since Kohler and Milstein first achieved their preparation in 1975 (Kohler and Milstein, 1975). The principle involved is: An antibody secreting cell (B-lymphocytes from immunized animal) produces only one particular antibody molecule and is not able to be grown in culture but if it is fused with a tumour cell with continuously growing and non-secreting characteristics, then the hybrid cell can secrete the desired antibody and be grown in culture and in vivo to produce large amounts of MAb.

Methods:

1. Preparation of immunogens

The immunogens used in immunizing rabbits and mice were Ptyr-KLH conjugate and purified DBP.

Coupling of phosphotyrosine (Ptyr) to a protein carrier KLH (Hemocyanin from Keyhole Limpets) or to BSA (Bovine serum albumin): 10μM (2.6mg) of O-phospho-L-tyrosine (Ptyr, Sigma) was dissolved in 1.8ml of 0.1M PBS (PH 7.5) and 10μM (3.1mg) of SPDP (N-Succinimidyl 3-(2-pyridyldithio) propionate, Pierce) was dissolved in 200μl of ethanol. The 200μl SPDP solution was added dropwise to the 1.8ml stirred phosphotyrosine solution to give a final concentration of 5mM of Ptyr-SPDP conjugate (Ptyr and SPDP were reacted at 1:1 molar ratio). The reaction mixture was left for half an hour at

room temperature with occasional stirring. 10mg KLH (Sigma) was dissolved in 3ml of 0.1M PBS at PH 7.5, then added into Ptyr-SPDP mixture and left for a further 2 hours. The reaction mixture was transferred to 4°C overnight and dialysed against PBS for 24 hours to remove excess reagent (Carlsson et al, 1978). Ptyr-BSA conjugate was prepared by the same procedure.

Alum precipitation of Ptyr-KLH and DBP for immunizing mice: In the case of Ptyr-KLH conjugate, 100µl of 1M $NaHCO_3$ (BDH) was added to 1ml of Ptyr-KLH (1mg/ml) and mixed by vortexing.100µl of 10% Aluminium Potassium Sulphate (Sigma) was added dropwise while vortexing. The mixture was pelleted at 500g for 10 min (MSE bench centrifuge) and the pellet was washed in PBS three times and resuspended in 1ml of sterile saline (Chase, 1967).

In the case of purified DBP (prepared by Ian Leith from Ad2 infected HeLa cells), 22.8ml of 0.25N NaOH was added dropwise to 10ml of 10% Aluminium Potassium Sulphate in a 50ml conical tube by vortexing. After 10 min incubation at room temperature, the mixture was centrifuged at 500g for 10 min (MSE) and the pellet was washed once with distilled water and resuspended in 10ml of sterile saline and added to 200µl of DBP (1µg/µl) in a 1.5ml Eppendorf tube. After incubation at room temperature for 30 min, the precipitate was pelleted in bench microcentrifuge (500g, 10 min) and resuspended in 1ml of sterile saline.

Adding Freund's adjuvants to Ptyr-KLH and DBP for immunizing rabbits: 1ml of Freund's complete or incomplete adjuvant was added into 1ml of Ptyr-KLH (1mg/ml) conjugate

or purified DBP (0.5mg/ml) in a 5ml Bijou and the mixture was emulsified by sonicating on ice.

2. Immunization of animals

Rabbits (Dutch) were immunized via the intramuscular route with 0.5mg of Ptyr-KLH conjugate or 0.25mg of purified DBP in an equal volume of complete Freund's adjuvant and boosted with the same amount of antigen in incomplete Freund's adjuvant at 3 week intervals 3 times. A final booster was given by intravenous injection without adjuvant. Three days after the final immunization, the serum after ear bleeding of the rabbit was tested by ELISA.

Mice (Balb/c) were immunized intraperitonally with 100μg of Ptyr-KLH or 20μg of purified DBP in the adjuvant of alum precipitate by the same procedure. Three days after final immunization, the sera from tail bleeding the mice were tested and the spleen was removed during the next 1-2 days from the mouse which gave the strongest serum response.

3. ELISA test on antisera (Catty and Raykundalia, 1989)

A 96-well plate was coated with ptyr-KLH and KLH alone or crude extracts from Ad2 infected Hela cells at the concentration of 10μg/ml overnight at 4°C and blocked with 200μl/well PBS containing 5% Marvel for half an hour at 37°C. Antisera from infected animals were double-diluted in PBS/5% Marvel applied to the plate at 50-100μl/well and incubated at 37°C for 1-2 hours. The plate was washed 2 times with PBS and followed by another hour's incubation of horseradish peroxides (HRP) labelled goat anti-rabbit (or mouse) IgG

(Sigma) diluted 1:1000-5000 in PBS/5% Marvel. After washing with PBS 5 times, 100µl of O-phenylenediamine dihydrochloride (OPD, Sigma) solution (4mg OPD/10ml 0.15M sodium citrate buffer at pH 5.6/5µl H_2O_2) was added to each well and the plate was left in the dark for 15-30 min to allow color developing. The results were visualized by eye or read at 492nm on a colorimetric plate reader.

4. Generation of hybrid cell lines

Preparing macrophages A mouse to be used was sacrificed in an ether jar and cleaned with 70% ethanol. 5ml of prewarmed (37°C) G-MEM (Glasgow modified Eagle medium)/10% NCS (newborn calf serum) was injected intraperitoneally into the mouse and the abdomen was massaged gently with two fingers at the sides. The organs of the mouse were rolled to the back and the inside fluid was squeezed from the sides to the middle and top of the abdomen and collected by piercing through the skin using a 18-G needle (the liquid dripped out to a tube). About 3-5ml of fluid (containing macrophages) was collected from one mouse. The macrophages were pelleted at 1200 rpm for 5 min (MSE bench centrifuge) and resuspended in 20-30ml HAT (hypoxanthine/aminopterin/thymidine, 50x, Sigma)/G-MEM/10% NCS.

Preparing myeloma cells Sp2/0 murine myeloma cells (Schulman et al, 1978) were maintained in G-MEM/10% NCS in a humidified CO_2 incubator at 37°C, being split 1:5 every 2-3 days. For fusion, the cells were grown to 90% confluent and changed to fresh medium one day before the fusion. Prior to fusion the cells were washed once with NCS-free G-MEM

medium, once with PBS then resuspended in 5ml of PBS and counted.

Preparing spleen cells spleens were removed from two mice and put into a boiling tube containing 5ml of PBS. The spleens were squeezed gently against the tube wall using a pestle to release the cells. After standing for 5 min, the suspension of spleen cells was transferred to a 10ml centrifuge tube using a pasteur pipette and the spleen cells were pelleted and resuspended in 5ml of 0.83% NH_4Cl to lyse the red cells. Set for 5 min, 3-4ml of calf serum was carefully underlaid and cells were pelleted again, then resuspended in 5ml of PBS and counted.

Fusion Myeloma cells were mixed with spleen cells at a cell ratio of 1:2-5 in a 50ml centrifuge tube and PBS added to 50ml. After spinning at 1200 rpm for 5 min, the supernatant was poured off and the tube was inverted to remove all the liquid. Tapped gently to loose the cells, 0.5ml of prewarmed PEG 1500 solution (50% of polyethylene glycol 1500 in 75mM Hepes at pH 7.5, Boehringer Mannheim) was added to the tube and mixed with cells by quickly centrifuging at 1200 rpm (taken up to 1200 rpm then turned down immediatly). After resuspending the cells, the tube was immersed in a 37°C water bath and held there for 20 sec with gentle swirling. 10ml of G-MEM/10% NCS was then slowly added in drops in 2 min time while the tube was swirling. Another 10ml of the same medium was added afterwards over 1 min. The fused cells were pelleted and resuspended carefully in 5ml of HAT medium. Mixed gently, sufficient HAT medium was added to make the concentration of cells at $2x10^5$ cells/ml. 100µl of

cell suspension was dispensed into each well of 96-well tissue culture plate in which 100μl of macrophage suspension had been added one day ahead. Peripheral wells of the plate were filled with PBS or H_2O. Hybrid cells were grown in HAT medium for 2 weeks after fusion then moved to HT (hypoxanthine/thymidine, Sigma) medium for a further 3-4 weeks before being maintained in medium (G-MEM/10% NCS).

5. Cloning and harvesting

Screening Screening for antibody-secreting hybridomas started about 2 weeks after and ELISA was chosen as a screening assay system for its simplicity and speed. For anti-Ptyr MAb, the Ptyr-BSA conjugate was used as a coating antigen. For anti-DBP MAb, Ad2 infected Hela cell extract (prepared as described in section I-6) was used as a coating antigen in early stage and purified DBP was used in late stage. 50μl of neat supernatant from a 10-50% confluent culture was applied to each antigen-coated well.

Subcloning All antigen-specific antibody-secreting hybridomas determined from ELISA were transferred with supernatant in neat to 1:10 dilution to a 24-well culture plate in which 0.5ml of macrophage suspension had been added to each well one day ahead. These hybridomas were tested again for their specific antibody secreting in about 5-7 days time (70%-90% confluent). Positive cultures were then subcloned to 96-well culture as follows (modified from Boeye, 1986): the number of cells per milliliter in the hybridoma culture was determined using a Burker cell counting chamber and a 1:10 series dilution was then made to a final density of 20 cells/ml, 100μl of

which was dispensed to each well (containing 100μl of macrophage suspension in nomal medium). After about 2-3 weeks, the hybridoma cells were screened again and the positive culture was subcloned to 96-well plate as before to ensure a single-cell clone.

Making ascitic fluid Specific antibody-secreting hybridoma cells were grown in 24-well plate to 90% confluent and washed twice with PBS, resuspended in 0.5ml of PBS and injected into mice to produce ascitic fluid which was collected after 2 weeks.

6. Characterization of antibodies by immunoassay

Cells, cell extracts and virus Hela spinner cells were grown in suspension in Glasgow S-MEM/7% NCS at 37°C and Hela monolayer cells were grown in G-MEM/10% NCS in a CO_2 incubator at 37°C. They were maintained by splitting at 1:5 every 3 days.

Ad2 infected and uninfected Hela cell extracts were prepared as follows from both spinner and monolayer cells depending on the requirement: 1000ml of Hela spinner cells (about $5x10^5$ cells/ml) or 1 flask (90% confluent) of Hela monolayer cells were infected with 20 p.f.u/cell of Ad2 in 1/10 original volume of S-MEM or G-MEM supplemented with 1% NCS. After 1 hour absorption at 37°C, MEM/1% NCS was added to original volume to maintain the cells. Where required, 0.5M hydroxyurea (Sigma) was added to 10mM at this stage to both infected and uninfected cells. At 12-72 hours post-infection (p.i.) the cells were pelleted, washed in PBS, resuspended in 1/100 original volume of lysis buffer (20mM Tris-HCl, 0.65M

NaCl, 1mM EDTA, 0.5% NP40, 1mM PMSF, 10μM $ZnSO_4$, and 50μM Na_3VO_4 at pH 7.5) and sonicated in ice. Cell lysates were clarified, aliquoted and stored at -70°C.

The adenovirus was grown in Hela spinner cells and extracted using Arcton as described previously (Russell et al, 1967; Winters and Russell, 1971).

SDS-PAGE Sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out at 150-200^{v} of constant voltage for 1-1.5 hours on a mini gel system (BIO-RAD) or at 70^{v} overnight on a big slab gel (Laemmli, 1970). The proteins to be analyzed were boiled (100°C, 3 min) in gel electrophoresis sample buffer (50mM Tris-HCl/2% SDS, 5% 2-mercaptoethanol/5% glycerol/0.015% bromophenol blue, at pH 7.0) and loaded (20-50μl) onto a 10% or 16% gel, which was a separating gel [10% or 16% of acrylamide : N,N'methylene-bisacrylamide (50 : 0.235 w/w) in 0.37M Tris-HCl/0.1% SDS/0.1% $(NH_4)_2S_2O_8$ (ammonium persulphate)/0.12% TEMED (N,N,N',N'-Tetramethylethylenediamine)/ at pH 8.6] overlayed with a spacer gel [5% of acrylamide : N,N'methylene-bisacrylamide (50 : 1.3 w/w) in 0.12M Tris-HCl/0.1% SDS/0.2% TEMED/0.1% $(NH_4)_2S_2O_8$, at pH 6.8]. After electrophoresis, the gel was either blotted to a nitrocellulose filter paper for western blotting or stained with PAGE-blue [2% PAGE-blue (BDH) in 10% acetic acid/30% methanol] and destained in acetic acid (10%)-methanol (30%) solution. ^{35}S labelled cell extracts were visualized by autoradiography of dried gel with Fuji X-ray film.

Western blotting Cell extracts and purified DBP were separated by SDS-PAGE and transferred to a nitrocellulose

filter paper at constant currents of 0.8mA/cm^2 gel for 1-2 hours using a semi-dry electroblotter (Pharmacia/LKB Biotechnology). From the anode (lower graphite plate of the blotter) to the cathode (upper U shape plate of the blotter), there were 6 layers of filter papers (Whatman) wetted in anodic buffer 1 (0.3M Tris-HCl/20% methanol at pH 10.4), 3 layers of filter papers wetted in anodic buffer 2 (25mM Tris-HCl/20% methanol at pH10.4), nitrocellulose filter paper (0.45μM, Schleicher&Schull) wetted in H_2O, SDS-PAGE gel washed once in H_2O and 9 layers of filter papers wetted in cathodic buffer (25mM Tris-HCl/40mM 6-amino-n-hexanoicacid/20% methanol at pH 9.4). After blotting, the nitrocellulose paper was washed once in PBS and blocked in 10% Marvel/PBS for 2 hours or overnight at room temperature. Washed twice with PBS/0.1% NP40, the membrane was incubated with an antiserum dilution in 5% Marvel/PBS for 1-2 hours. After this, the membrane was washed twice with PBS/0.1% NP40 and incubated with either a secondary antibody labelled with HRP (horseradish peroxidase, Sigma) followed by color developing with CNP solution (15mg 4-chloro-1-naphthol / 5ml methanol/20ml PBS/15μl 30% H_2O_2) or ^{125}I labelled protein A (Amersham) followed by autoradography.

Immunofluorescence A431 and Hela cells were grown on multispot miroscope slides in G-MEM/10% NCS in petri dishes to 90% confluent. Then, A431 cells were stimulated or mock-stimulated with 100-200ng/ml of epidermal growth factor (EGF) to activate membrane epidermal growth factor (EGFR), and Hela cells were infected or mock-infected with 20

p.f.u/cell Ad2. After 0.5-1 hours incubation with EGF (A431 cells) and 3-48 hours postinfection (Hela cells) at 37°C (CO_2 incubator), the two cells were washed twice in PBS and fixed in Formaldehyde (5% Formaldehyde/2% Sucrose/PBS) for 10 min. The cells were washed three times in PBS containing 1% newborn calf serum (NCS) and then penetrated with NP40 (0.5% NP40/10% Sucrose/1% NCS/PBS) for 5 min followed by three times washing in PBS/1% NCS. Antisera or ascitic fluid were diluted in PBS/1% NCs in series and spotted onto each well of the slides. After incubation at 37°C in a humidified atmosphere for 1 hour, the slides were washed in PBS/1% NCS and 1 drop of a 1:100 dilution of FITC (fluorescein isothiocyanate) labelled anti-rabbit globulin (Sigma) or Texas Red labled anti-mouse globulin (Sigma) were added to each well. After incubation (0.5-1 hours, 37°C) and washing (3-5 times in PBS/1% NCS), the slides were covered and examined in a fluorescence microscope. FITC is absorbed at 495nm generating an apple-green color and Texas Red is absorbed at 596nm generating a red color.

Proteolytic digestion of DBP (Cleghon and Klessig, 1992) 5μg of purified Ad2 DBP was digested with 0.03μg-2μg of chymotrypsin (Sigma) and Trypsin (Sigma) at weight ratios of protease : DBP of 1:160, 1:40, 1:10 and 1:2.5. The proteolytic digestion reaction was carried out in 25mM Tris-HCl (pH 8.0)/1mM EDTA/100mM NaCl in a volume of 45μl at room temparature for 20 min. After that, 15μl of 4x SDS PAGE sample buffer was added into each reaction mix and proteolysized DBP fragments were denatured at 100°C for 3

min, separated by SDS PAGE, blotted into nitrocellulose filter paper and probed with anti-DBP antibodies.

7. Purification of antibodies

Purifying polyclonal anti-Ptyr by Affi-Gel 15 1.5ml of Affi-gel (BIO-RAD) were washed throughly with cold deionized H_2O and mixed with 3ml of Ptyr (O-phospho-L-tyrosine, Sigma) overnight at 4°C on a rocker (Frost et al, 1981). Ptyr was prepared in 50mM Tris-HCl/150mM NaCl pH 7.5 at 25mg/ml. 150µl of 1M ethanolamine-HCl (pH 8.0) was added to above coupling mix to block any active ester in the gel. After incubation at 4°C for a further 1 hour, the gel was washed with H_2O as before and equilibrated in 50mM Tris/150mM NaCl (pH 8.0). 10ml of rabbit antiserum to Ptyr were mixed with the gel overnight at 4°C on a rocker. After which the gel was transferred to a 2ml disposable column and washed with 40ml of 5mM phosphoserine/5mM phosphotheorinine in 5mM sodium phosphate pH 7.2 (Kamps and Sefton, 1988). The anti-Ptyr antibody was eluted with 0.1M Glycine-HCl at pH 2.8 and collected in 200µl fractions in Eppendorf tubes containing 20µl (1/10 volume) of 1M Tris pH 7.5. 3µl of each fraction was spotted onto a nitrocellulose paper and the paper was stained with 1% napthalene black and destained in 10% acetic acid/30% methanol. Peak fractions were pooled and dialysed against PBS.

Purifying monoclonal anti-DBP by protein G-Sepharose 1mg of protein G-Sepharose 4B fast flow beads (in suspension in 20% ethanol, Sigma) were washed well with PBS and mixed with 2ml of ascitic fluid (clarified prior to mixing) overnight at

4°C with gentle rocking. Then the gel was loaded onto a column and washed with 10-20 bed-volumes of PBS and the MAb was eluted with 0.1M Glycine-HCl/0.1M NaCl at pH 2.8 in 0.5ml fractions, neutralized with 50μl (1/10 volume) of 1M Tris-HCl pH 7.5. Peak fractions (estimated as above) were pooled. Half of it was dialysed against PBS and half of it was dialysed against coupling buffer (0.1M Borate/0.5M NaCl) for making affinity column.

Results:

1. Preliminary test on antisera

An antiserum from the rabbit immunized with the ptyr-KLH conjugate was double-diluted and applied to an ELISA plate which had been coated with ptyr-KLH (immunogen) and KLH (the protein carrier) (Fig.I-1. Lanes 3, 4 and 5). To make a comparison, the serum was also neutralized by KLH before being diluted out and applied to the plate (Fig.I-1. Lanes 8, 9 and 10). Figure I-1 showed that the serum presented positive in both KLH-coated and (ptyr-KLH)-coated wells before being neutralized by KLH but only in (ptyr-KLH)-coated wells after neutralization with KLH, suggesting a specific antibody against phosphotyrosine had been raised in the rabbit. Using the same conjugate, several mice were immunized and similar results were obtained from these sera by the same method of testing (Fig.I-2).

Anti-sera to Ad2 DBP from the rabbit and mice were tested by ELISA in a similar procedure (data not provided). The 96-well plate was coated with Ad2 infected and mock-

infected Hela cell extracts. To confirm the result, mice anti-sera were also tested by western blotting (Fig.I-3) before a fusion was carried out. From figure I-3, it is suggested that the positive response which appeared in the ELISA plate was specific to DBP since corresponding positive bands appeared in western blots were presented in the Ad2 infected cell extracts (Fig.I-3. Lanes 2 and 3) but not the mock-infected cell extracts (Fig.I-3. Lane 4).

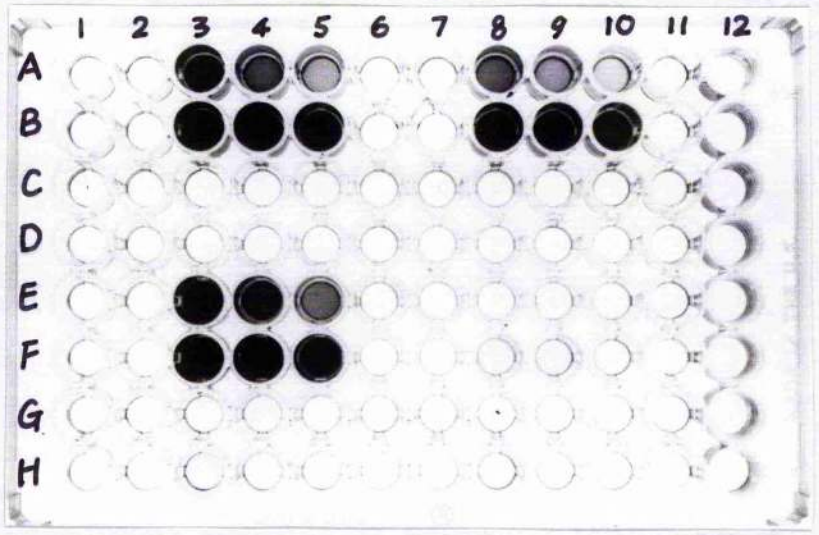


Fig.I-1. An ELISA test for the specificity of the rabbit antiserum to phosphotyrosine. Rows A, B and C, D were coated with ptyr-KLH; Rows E, F and G, H were coated with KLH alone. The anti-serum was applied in double-dilutions as follows: B3 & B8: 1:1000; B4 & B9: 1:2000; B5 & B10: 1:4000; A3 & A8: 1:8000; A4 & A9: 1:16000; A5 & A10: 1:32000. Similarly, wells F3 & F8 to E5 & E10 were dilutions of 1:125 to 1:4000. Samples in columes 8, 9 and 10 were neutralized by KLH prior to being applied to each well. Samples in rows C, D and G, H were corresponding dilutions of an antiserum from the rabbit which had not been immunized by ptyr-KLH.

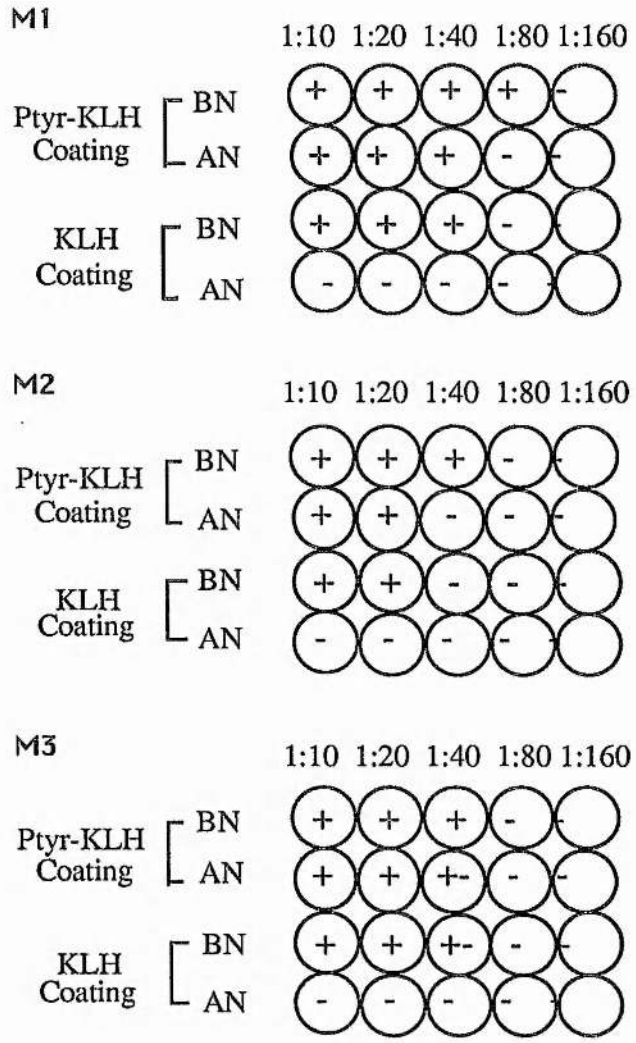


Fig.1-2. An ELISA test of antisera to phosphotyrosine from the mice immunized with the ptyr-KLH conjugate. M: mice BN: before neutralization; AN: after neutralization.

2. Production of antibodies

Anti-Ptyr MAbs Several fusions were performed using the spleens removed from immunized mice responding positively to phosphotyrosine on their sera. About 2% of the supernatants on average were positive (reacted with Ptyr-KLH) in an ELISA test. From the first two fusions, all positive

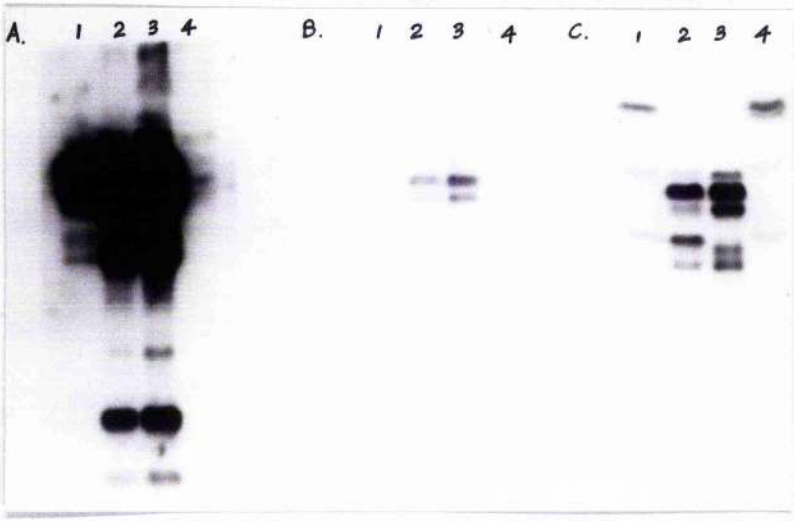


Fig.I-3. A western blot of Ad2 infected and mock-infected Hela cells extracts with mice anti-sera to DBP. A: a known anti-serum to DBP from a guinea pig; B and C: anti-sera from 2 mice immunized with puified DBP. Lanes 1, 2, and 3: Ad2 infected Hela cell extracts; Lane 4: Ad2 mock-infected Hela cell extracts.

supernatants appeared to react with KLH (carrier) but not with Ptyr since they presented a positive result in both Ptyr-KLH and KLH coated plates. This probably resulted from a poor conjugate (see discussion). After modification of conjugate preparation by increasing the time allowed for the reaction between Ptyr-SPDP and KLH to 1 hour (see discussion), further immunization and fusion were performed and specific antibodies against Ptyr were found in some hybridoma cells. Thus subcloning rescreening and resubcloning were carried out, and finally three MAbs were isolated and harvested as ascitic fluid. Surprisingly, when these three MAbs had been characterized they were found to be MAbs to BSA but not to Ptyr. BSA was used as a carrier protein of Ptyr in all screening assay (i.e. ELISA plate was coated with Ptyr-BSA). In a phosphatase activity test (standard p-nitrophenol phosphate assay, Sigma), both BSA and KLH appeared to be positive and BSA was stronger

(data not provided). This suggests that the phosphate in the phosphotyrosine linked to BSA had been taken off gradually by a trace of phosphatase in the carrier protein during the long process, leading to a selection of MAbs to BSA (see discussion).

Anti-DBP MAb Two fusions have been performed for anti-DBP MAbs. About 4% of the supernatants were presented positive in the first screening. Only 20-30% of the positive hybridoms remained positive in the second screening (i.e. after first subcloning). For the first fusion, anti-DBP-secreting hybridoma cells died gradually during the first and second subcloning. For the second fusion, however, two anti-DBP-secreting hybridomas 12B8 and 15C2 were isolated and anti-DBP MAbs were harvested as ascitic fluid. The ascitic fluid of 15C2 appeared not to react with DBP since in an ELISA test, the ascitic fluid from 12B8 has a high titre in reacting with DBP while the ascitic fluid from 15C2 has a negative response to DBP.

3. Characterization of antibodies by immunoassay

Polyclonal anti-Ptyr recognizes EGFR in A431 cell membranec

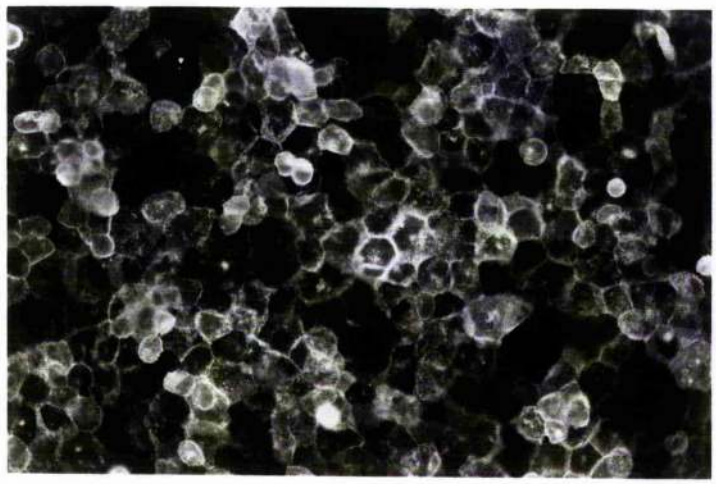
A431 is a human epidermoid carcinoma cell line which has been identified to have an unusually high number of specific epidermal growth factor receptors (EGFR) in its membrane (Wrann and Fox, 1979). The EGFR has a tyrosine kinase activity and is autophosphorylated when stimulated with epidermal growth factor (EGF) at physiological concentrations (Hunter and Cooper, 1981). Thus A431 cells were used in antibody identification as a positive control substrate for tyrosine

kinase and in investigating tyrosine phosphorylation on DBP as both a substrate and a kinase (see part II).

In a immunofluorescence assay, A431 cells were grown on slides to 90% confluent, stimulated and mock-stimulated with 100ng/ml of EGF, and probed with a 1 : 200 dilution of rabbit antiserum against Ptyr. Commercial anti-Ptyr MAb PT-66 (Sigma) was used (1:100) as a positive control. A rabbit antiserum to DBP and an unrelated ascitic fluid Bio/4 were used as negative controls. After immunostain with FITC or Texas Red linked secondary antibody, bright fluorescence appeared only in EGF stimulated A431 cell membrane that had been probed with polyclonal anti-Ptyr (Fig.I-4. A.) and monoclonal anti-Ptyr antibodies (data not shown), suggesting that the rabbit polyclonal anti-Ptyr serum specifically recognizes native phosphotyrosine-containing protein as commercial MAb to Ptyr does. As a comparison, Figure I-4 shows the result of EGF stimulated and unstimulated A431 cells probed with the rabbit antiserum to Ptyr. From Fig. A and B, it can be seen clearly that there is an significant increase in the phosphotyrosine level in membranes between stimulated and unstimulated cells.

For a western blotting assay, A431 cells were grown in 8cm dishes, stimulated with EGF in vivo and in vitro (see part II methods), separated by SDS PAGE, transferred to nitrocellulose filter paper and probed with the same antibodies as used in an immunofluorescence assay described above. Specific bands appeared in the blots that were probed with anti-Ptyr rabbit serum (Fig.I-5. A) and MAb PT-66 (Fig.I-

A.



B.

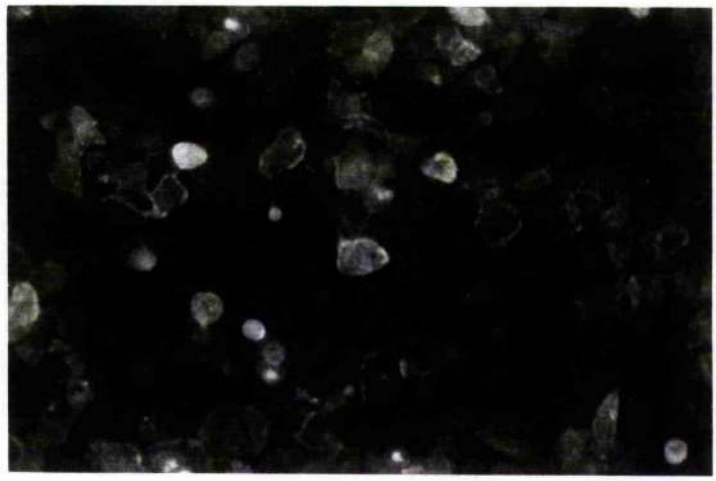


Fig.I-4. Immunofluorescence assay on A431 cells stimulated with EGF (A) or without EGF (B) followed by probing with the rabbit anti-Ptyr antibody.

5. C), suggesting that anti-Ptyr rabbit serum recognized a denatured phosphotyrosine-containing protein. To in vitro EGF stimulated A431 cell membrane extracts, anti-Ptyr rabbit serum and MAb PT-66 reacted in the same way (Fig.I-5. Lanes

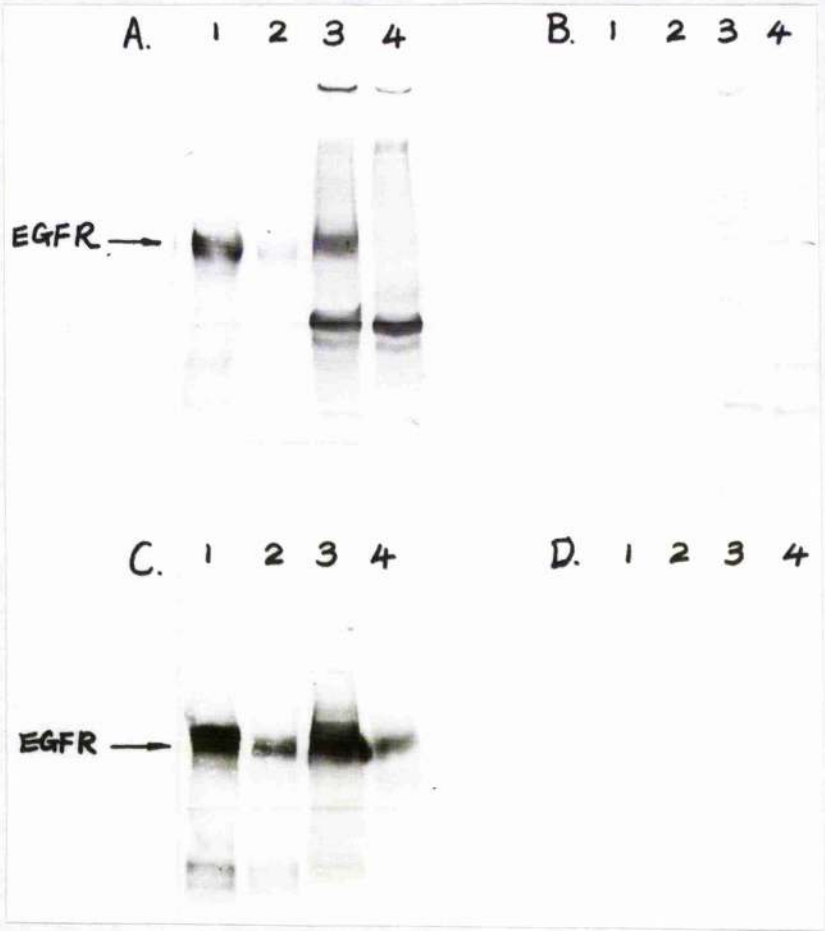


Fig.I-5. Western blotting on EGF stimulated and mock-stimulated A431 cell membrane extracts probed with antibodies against Ptyr. Lanes 1 (and 2): EGF in vitro stimulated (and mock-stimulated) A431 cell membrane extracts; Lanes 3 (and 4): EGF in vivo stimulated (and mock-stimulated) A431 cell membrane extracts. A. anti-Ptyr rabbit serum (1:250); B. control rabbit serum (anti-DBP, 1:250); C. anti-Ptyr MAb PT-66 (1:100); D. control mouse ascitic fluid Bio/4 (1:100).

1 and 2 in A. C.). To in vivo EGF stimulated cell membrane extracts, MAb PT-66 also recognized EGF mock-stimulated EGFR (containing lower lever of phosphotyrosine) but anti-Ptyr rabbit serum did not (Fig.I-5. Lanes 4 in A. C.); whereas anti-Ptyr rabbit serum picked up an extra phosphotyrosine-

containing protein with lower molecular weight in both EGF stimulated and unstimulated cell membrane extracts but MAb PT-66 did not (Fig.I-5. Lanes 3 and 4 in A. C.), suggesting that although anti-Ptyr rabbit serum can recognize denatured phosphotyrosing-containing protein as PT-66 does, it behaves differently from PT-66.

MAb 12B8 recognizes native and denatured forms of both viral DBP and expressed DBP

To test whether anti-DBP MAb (12B8) recognizes denatured DBP and DBP expressed in Baculovirus. Ad2 infected and uninfected Hela cell extracts, purified viral DBP (V-DBP) and purified Baculovirus DBP (B-DBP) were absorbed to a 96-well ELISA plate and an ELISA was performed. The same protein samples were also run on a SDS gel and transferred to a membrane which was then probed with different MAbs. Two known anti-DBP MAbs B6 and A1 (Reich et al, 1983) were used for comparison. B6 recognized both native and denatured forms of DBP whereas A1 only recognized the native form of DBP. All MAbs were used at a 1:500 dilution in western blotting. In ELISA MAbs were applied in a dilution series (starting at 1:100) but only OD readings from a 1:500 dilution (same as used in western blotting) of each MAb at 492nm were selected and presented with corresponding blots (Fig.I-6). From figure I-6, it can be seen that anti-DBP 12B8 specifically recognized DBP in crude cell extracts (Fig.I-6. A. Lanes 1 and 2), purified form of wild type virus (Fig.I-6. A. Lane 3) and recombinant baculovirus (Fig.I-6. A. Lane 4).

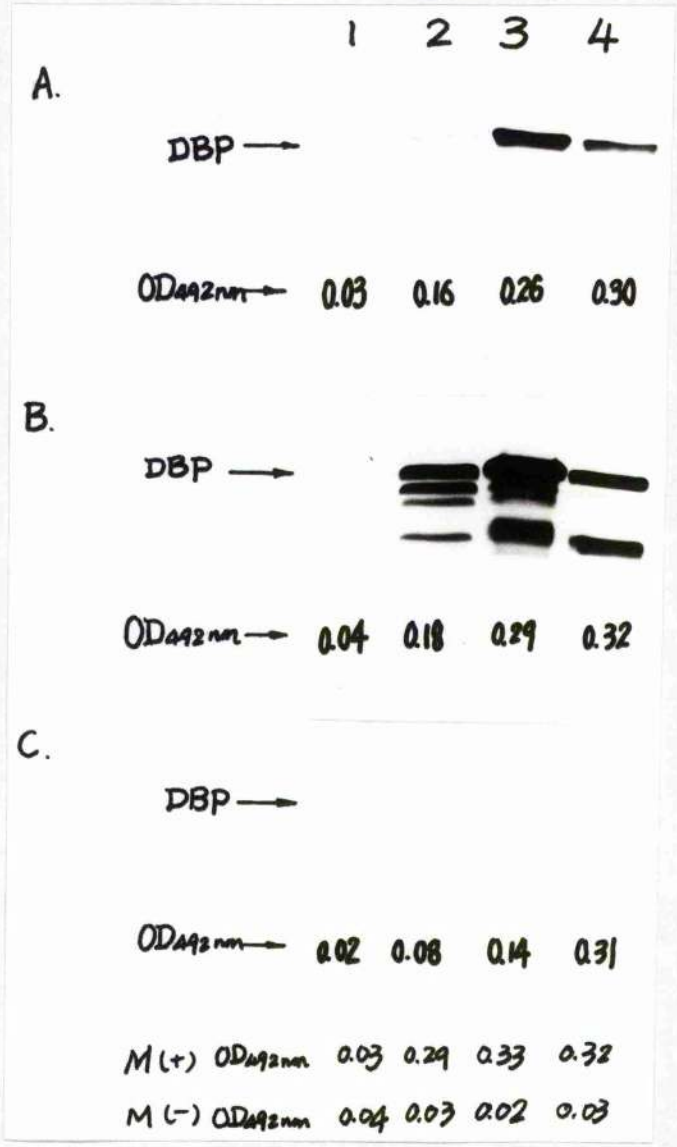


Fig.I-6. ELISA and Western blotting on MAbs to Ad2 DBP. A. 12B8; B. B6; C. A1. Lane 1: uninfected Hela cell extracts; Lane 2: Ad2 infected Hela cell extracts Lane3: B-DBP Lane 4: U-DBP

<u>MAb 12B8 is not a protein A binder</u> To determine whether MAb 12B8 is a protein A binder or not, 2 membranes (blotted with DBP) were probed with 12B8. Then one of them was incubated with ^{125}I labelled protein A followed by autoradiography and the other was incubated with an anti-

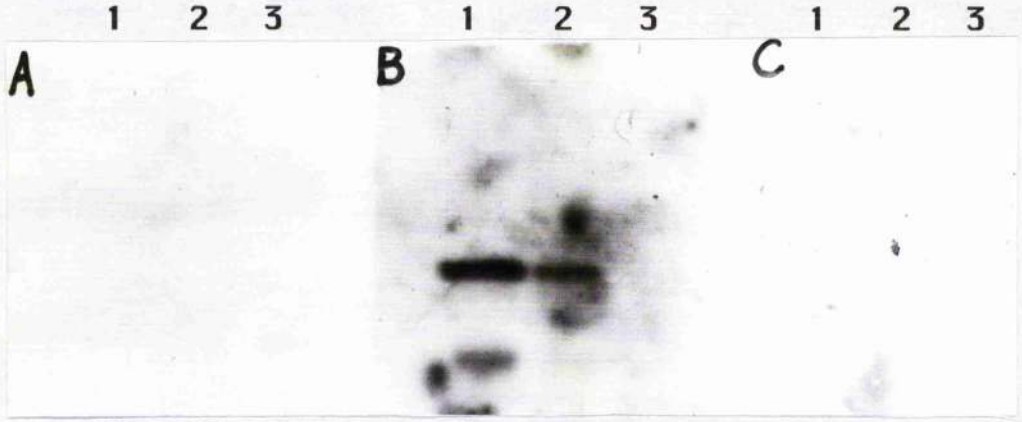


Fig.I-7. An autoradiography of western blots probed with MAb 12B8 followed by: A. incubating directly with ^{125}I labelled protein A; B. with rabbit anti-mouse IgG prior to the addition of ^{125}I labelled protein A. C. negative control: same as B. but the membrane was probed to a negative control ascitic fluid Bio/4.

mouse rabbit immunoglobulin before addition of ^{125}I labelled protein A. After autoradiography DBP bands appeared in the membrane incubated with rabbit antiserum to mouse immunoglobulin prior to the addition of protein A (Fig.I-7. B), but not in the membrane incubated with protein A directly (Fig.I-7. A), suggesting that anti-DBP MAb 12B8 is not a protein A binder.

<u>MAb binding to protease-digested DBP fragment</u> To identify which proteolytic fragment of DBP reacts with MAb, purified DBP was digested with chymotrypsin and trypsin, separated by SDS PAGE, blotted to nitrocellulose filter paper and probed with both MAb and PAb (polyclonal antibody) to DBP. Surprisingly, after intensive treatment with proteases, there was no significant difference in Ag (antigen)-Ab (antibody) interaction pattern between MAb and PAb in western blotting (Fig.I-8). This suggests that DBP may contain only a few linear

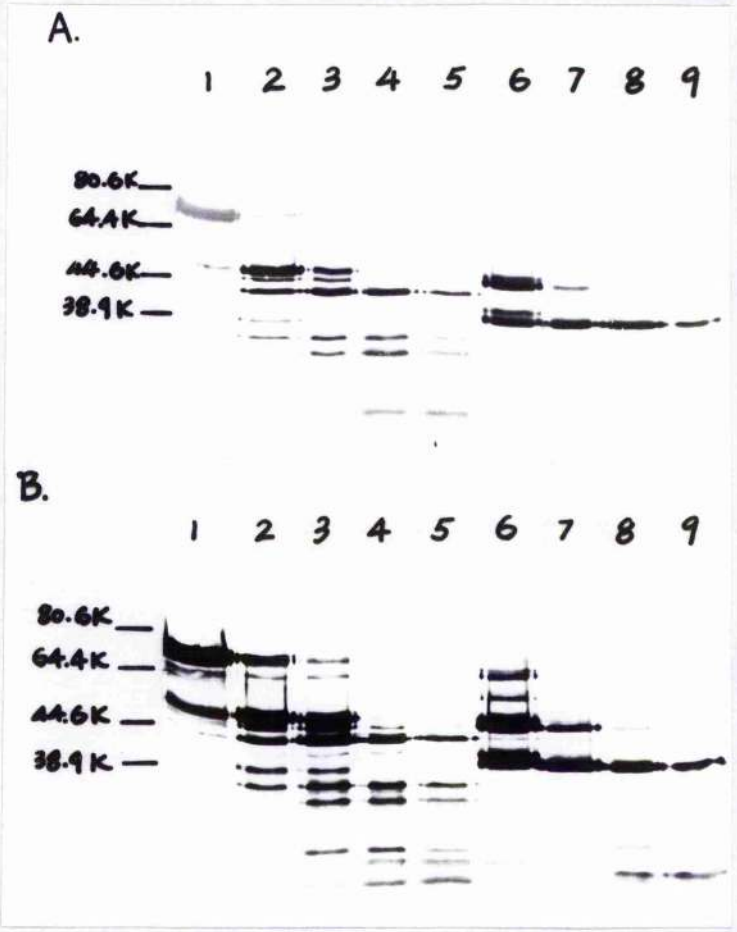


Fig.I-8. Western blotting of proteolytic digests of purified DBP (5μg). Lane 1: untreated purified DBP; Lanes 2-5: chymotrypsin treated DBP (0.03μg, 0.125μg, 0.5μg and 2μg protase per digestion respectively); Lanes 6-9: trypsin treated DBP (0.03μg, 0.125μg, 0.5μg and 2μg respectively). A. The blot was probed with MAb 12B8; B. The blot was probed with rabbit PAb

antigenic sites and the MAb 12B8 probably recognized the major linear antigenic site of the protein. By chymotrypsin digestion, one major band appeared in both blots (Fig.I-8, A and B, Lanes 4 & 5) just below the molecular weight marker of 44.6K (Fig.I-8). By trypsin digestion, it can be seen more clearly that almost only one band appeared in blots (Fig.I-8, A and B, Lanes 8 & 9) and it has an apparent molecular weight

of about 39K (Fig.I-8). Do these results suggest that the antigenic site recognized by the MAb 12B8 is located at the C-terminal of the protein? If this is true, the MAb would be very useful in isolating DNA binding sites of the DBP in this domain. To confirm this, further experiments are required.

4. Purification of antibodies

Rabbit antiserum to Ptyr was purified using an Affi-Gel 15 column which had been coupled with Ptyr (see methods). Peak fractions eluted from the column were pooled and tested for its purity and function. By dot blotting, the antiserum load , flow-through and elute were double-diluted (starting at 1:10) and spotted (3μl) to a membrane, which had been coated with Ptyr-KLH, and incubated with ^{125}I labelled protein A for 15 min followed by washing and autoradiography (Fig.I-9). Here, antiserum "load" stands for the fraction of the antiserum that has not been applied to the column [i.e. anti-(Ptyr-KLH)]; "flow-through" stands for the fraction of the antiserum that had been applied to the column and passed through the column (i.e. anti-KLH portion of the antiserum); and "elute" stands for the fraction of the antiserum that had been applied to the column, bound to the column and eluted from the column by low pH buffer (i.e. anti-Ptyr portion of the antiserum). From figure I-9, it shows that the majority of antibodies in the antiserum (before purification) were anti-KLH (Fig.I-9. row B), anti-Ptyr antibody was only a small portion (Fig.I-9. row C). Whether this small portion of anti-Ptyr antibody had been fully

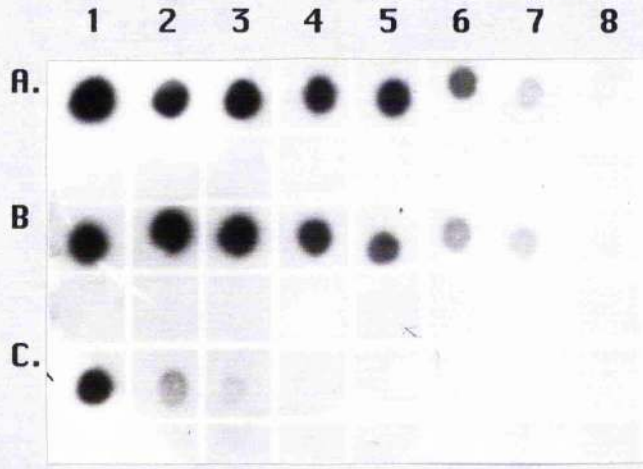


Fig.I-9. An autoradiograph of a Dot blot. The conjugate of Ptyr-KLH was absorbed to a nitrocellulose filter paper on which row A was spotted with antiserum load [anti-(Ptyr-KLH)], row B was spotted with antiserum flow through (anti-KLH) and row C was spotted with antiserum elute (anti-Ptyr). Lanes 1-8 were doubling-dilutions of each antiserum from 1:10 (Lane 1) to 1:1280 (Lane 8).

separated from the majority anti-KLH antibody or not, these antiserum fractions (load, flow-through and elute) were further examined by an immunoprecipitation assay using phosphotyrosine-containing A431 cell extracts, since the phosphotyrosine-containing protein used in Dot blotting was the immunogen of phosphotyrosine linked KLH (Ptyr-KLH).

For Immunoprecipitation, A431 cell membrane extracts and solubilized extracts were stimulated with EGF, labeled with ^{32}P and immunoprecipitated (see part II methods) with the antiserum load [anti-(Ptyr-KLH)], flow-through (anti-KLH) and elute (anti-Ptyr). The result presented in Fig.I-10 shows that tyrosine-phosphorylated EGFR (in A431 cell extracts) was immunoprecipitated by antiserum load and eluate (Fig.I-10. Lanes 1, 3, 4, 8, 10 and 11) but not by flow-through (Fig.I-10.

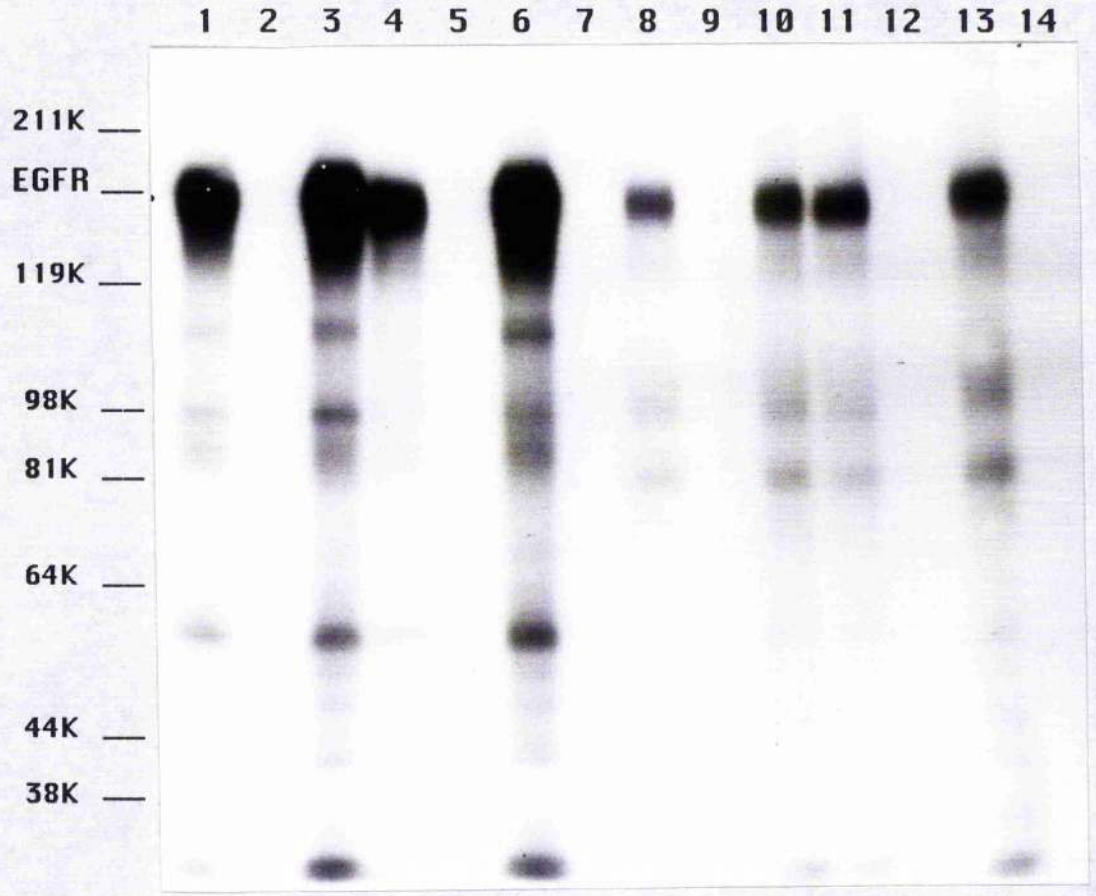


Fig.I-10. An autoradiography of EGF stimulated A431 cell extracts immunoprecipitaed with antiserum fractions (load, flow- through and elute) after the extracts have been labeled with ^{32}P in vitro. Lanes 1-7 were solubilized extracts and lanes 8-14 were membrane extracts. Lanes 1 and 8: antiserum load [anti-(Ptyr-KLH)]; Lanes 2 and 9: antiserum flow-through (anti-KLH); Lanes 3&4 and 10&11: antiserum eluate (anti-Ptyr); Lanes 5&12: control rabbit antiserum (anti-SV5); Lanes 6&13: commercial anti-Ptyr MAb PT-66; Lanes 7&14: control ascitic fluid of Bio/4.

Lanes 2 and 9), suggesting that antiserum that has been applied to and passed through the column does not contain any anti-Ptyr antibody. Anti-Ptyr antibody has been completely separated from anti-KLH antibody in the rabbit serum and purified anti-Ptyr antibody functioned as before in

immunoprecipitation since it had been denatured during purification.

To check the purity and to estimate the concentration of the purified antibodies, purified anti-Ptyr polyclonal antibody and anti-DBP MAb were analysed by SDS PAGE, using different concentrations of BSA as standards (Fig.I-11). Figure I-11 shows that only one heavy chain and one light chain appeared in both antibodies (Fig.I-11. Lanes 4, 6 and 7), suggesting the two antibody products are pure, and the estimated concentration of anti-Ptyr antibody is 2mg/ml and anti-DBP antibody is 1mg/ml (Fig.I-11).

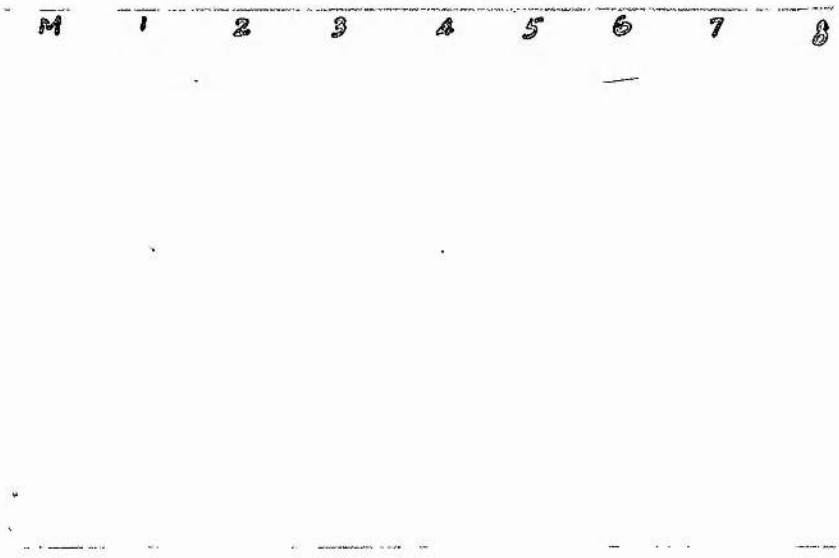


Fig.I-11. SDS PAGE of purified antibodies. M: molecular weight marker: 205K, 116K, 97K, 66K, 45K and 29K. Lanes 1-3: BSA of 2mg/ml, 1mg/ml and 0.5mg/ml respectively; Lane 4: purified anti-Ptyr polyclonal antibody; Lane 5: Ptyr-column wash; Lane 6 and 7: purified anti-DBP MAb in PBS and coupling buffer respectively; Lane 8: protein G column wash.

Discussion:

1. Failure to make anti-Ptyr MAb

Phosphotyrosine-containing proteins can be detected by autoradiography of gels treated with alkali since phosphotyrosine is more alkaline resistant than phosphoserine and phosphothreonine (Cooper et al, 1983). But it is not an ideal method to examine phosphotyrosine in proteins not only because of its high background and low sensitivity. Most importantly, not all of the phosphoserines and phosphothreonines are hydrolyzed during alkaline treament (Yin and Wang, 1988). We found it very difficult to achieve satisfactory results detecting phosphotyrosine by alkaline treatment. Ross et al first developed antibodies for phosphotyrosine using p-azobenzyl phosphonate (ABP) (Ross et al, 1981). This approach now has been well extended and used in studying protein tyrosyl-phosphorylation. Although commercial antibodies against phosphotyrosine are available the extremely high price make it impossible to use it in multiple experiments. Therefore, it was decided to make our own anti-phosphotyrosine antibodies to study the DBP tyrosyl-phosphorylation. Because phosphotyrosine is too small to induce an immune response, various methods have been used to couple phosphotyrosine (or its analogs) to a protein carrier to generate effective immunogens. We used the heterobifunctional reagent N-succinimicyl 3 (2-pyridyldithio) propionate (SPDP) as a linker, and Keyhole limpet hemocyanin (KLH) as a protein carrier to prepare the conjugates of Ptyr-KLH and Ptyr-BSA according to the method

of Carlsson et al (Carlsson et al, 1978). The prepared conjugate of Ptyr-KLH has been used to immunize a rabbit and mice and generated antibodies against phosphotyrosine (Fig.I-1, I-2). The prepared conjugate of Ptyr-BSA has been used in a screening assay of ELISA as a coating antigen. The quality of the conjugates and the method used in the screening assay directly affected the production of MAb.

Generation of a poor conjugate N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) is a heterobifunctional reagent. It contains one N-hydroxysuccinimide ester moiety and one 2-pyridyl disulphide moiety. The hydroxysuccinimide ester reacts with the amino group of Ptyr firstly to give a stable amide bond, and the 2-pyridlyl disulphide group reacts with aliphatic thiols of KLH or BSA afterwards. In aqueous form, the N-hydoxysuccinimide ester of SPDP is decreased quickly because of hydrolysis. In ethanol it is stable (Carlsson et al, 1978). If there are amino groups in the media, the aminolysis of N-hydroxysuccinimide ester is much faster than the hydrolysis (Fig.I-12). In the first step of coupling, Ptyr-SPDP was formed by aminolysis of N-hydroxysuccinimide ester of SPDP with amino group of Ptyr, so it is important to prepare the SPDP solution in ethanol and dilute it (if

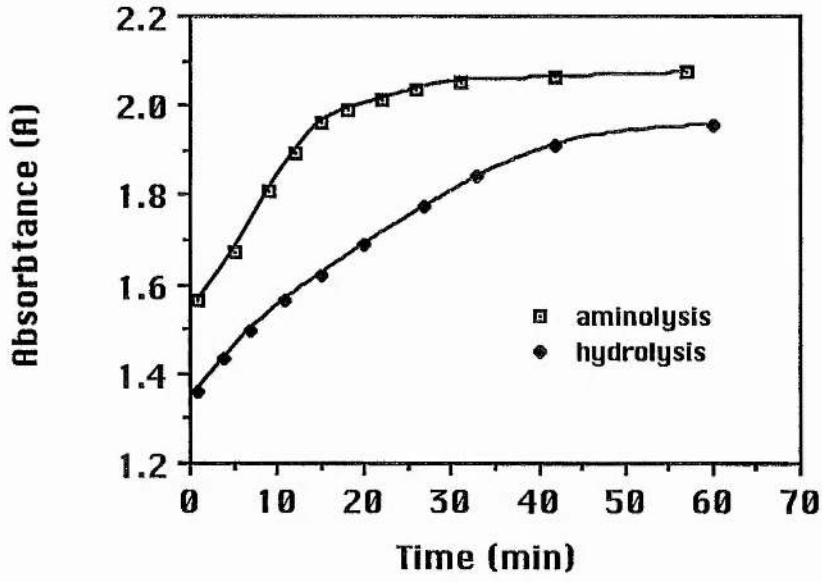
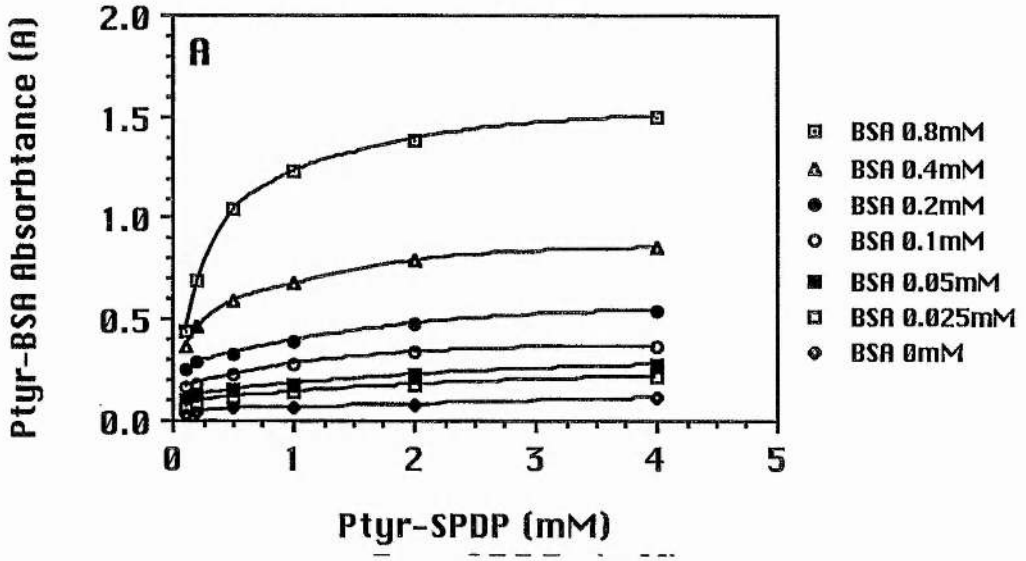


Fig.1-12. SPDP aminolysis and hydrolysis by measuring the absorbance of the N-hydroxysuccinimide released from both aminolysis and hydrolysis of SPDP at λ_{max}=260nm. Methodology was as described in the Pierce handbook, spectrophotometry was carried out using a Perkin Elmer lambda 5. The aminolysis of SPDP takes about 20 min whereas the hydrolysis of SPDP takes about 40 min.

necessary) just before use or directly into the reaction mixture to avoid the hydrolysis of hydroxysuccinimide ester before it reacts with Ptyr. It is also important to control the molar ratio of Ptyr/SPDP to be 1 or greater than 1 to avoid the excess of SPDP (SPDP and Ptyr contain one hydroxysuccinimide ester and one amino group respectively), since the N-hydroxysuccinimide ester of excess SPDP would be hydrolyzed whereas the 2-pyridyl disulphide moiety of excess SPDP would take part in the next reaction to generate a conjugate of linker and carrier. Such a conjugate would only generate anti-KLH (carrier) antibody.

In the second step of coupling, the extent of thiol-disulphide exchange of the 2-pyridyl disulphide group in SPDP and the aliphatic thiols in BSA (or KLH) depends on the amount of Ptyr-SPDP and BSA (or KLH) to be used (Fig.1-13). It is expected that the protein carrier molecule could be surrounded by as many Ptyr molecules as possible (i.e. a high degree of substitution). Thus an excess of Ptyr-SPDP is required. In contrast, an excess of the protein carrier is avoided. It would result in a poor conjugate (with low degree of substitution) and generate an antibody to carrier. It can be seen that when the content of Ptyr-SPDP is greater than 0.5mM, the product of Ptyr-BSA does not increase with Ptyr-SPDP (Fig.1-13. A); it increases linearly with BSA (Fig.1-13. B). This suggests that the 2-pyridyl disulphide groups have been saturated. When the concentration of Ptyr-SPDP is in the range of 0.5mM-4mM, the concentration of BSA could change from 0.1mM (6.8mg) to 0.8mM (54.4mg) and the relationship between Ptyr-BSA and BSA remains linear (Fig.1-13. A). Thus in

FIG.1-13.



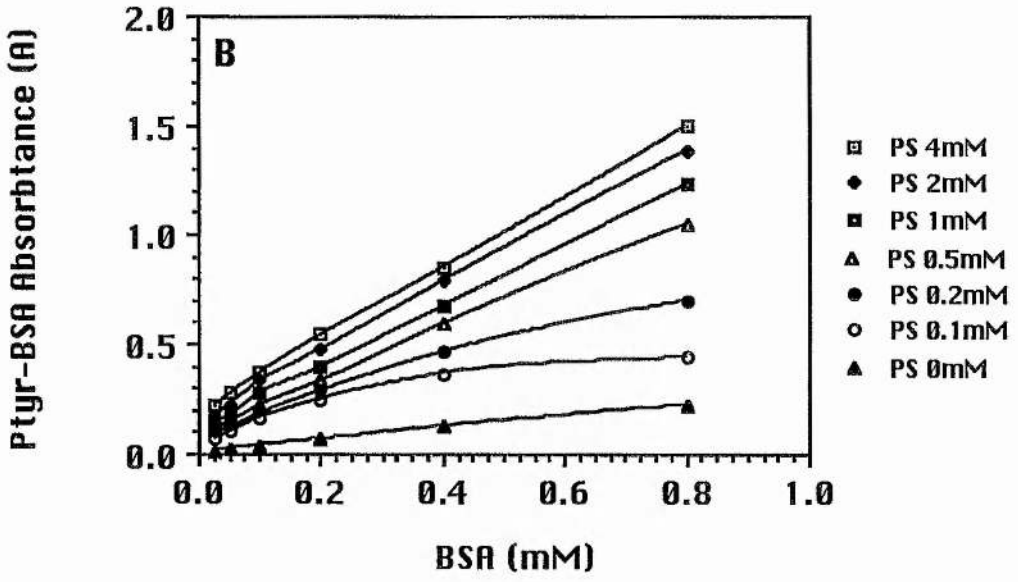


Fig.I-13. Thiol-disulphide exchange of the 2-pyridlyl disulphide group in SPDP and the aliphatic thiols in BSA determined by measuring the absorbance of the pyridine-2-thione released from the thiol-disulphide exchange reaction at λ_{max}=343nm. A) Absorbance of Ptyr-BSA as a function of Ptyr-SPDP; B) Absorbance of Ptyr-BSA as a function of BSA.

the case of 5mM Ptyr-SPDP and 10mg BSA (or KLH) which were used in coupling reaction, there are plenty of 2-pyridyl disulphide groups to be used in exchange reaction. A poor conjugate is unlikely to be generated by an excess of the protein.

It should be pointed out that the time of thiol-disulphide exchange reaction varies from 10 min to 3.5 hours depending on the amount of protein being used (Fig.I-14. A). Obviously, if the reaction is stopped by dialysis process, a poorer conjugate (lower degree of substitution) is thus generated. It takes about 1 hour to finish the reaction of thiol-disulphide exchange for BSA at 0.2mM (13.6mg) (Fig.I-14. B). Only 40 min

was used in thiol-disulphide exchange reaction in preparing the conjugate which had been used in immunizing the mice for the first 2 fusions.

Selection of antibody-secreting hybridomas In the screening assay of ELISA, Ptyr-KLH and KLH were first used as coating antigens and MAbs were selected indirectly by reacting with Ptyr-KLH conjugates (immunogen) and KLH (protein carrier). Only the one which has a positive reaction with Ptyr-KLH and a negative reaction with KLH would be selected. This method doubled labour and did not provide clear results. Thus BSA, an unrelated protein, was used in coupling of Ptyr to form a conjugate of Ptyr-BSA, and the Ptyr-BSA conjugate was then used as a coating antigen in ELISA in place of Ptyr-KLH & KLH throughout the process. In a phosphatase activity assay, both KLH and BSA appeared positive, suggesting that they contained phosphatase (even a trace amount, phosphatase contamination would be a disaster in this application). Ptyr-KLH and Ptyr-BSA were prepared at the same time and stored in 4°C. For immunization of the mice, Ptyr-KLH was used in the first 6-7 weeks after preparation while for the screening of hybridoma cells, Ptyr-BSA was used two months later after preparation. During this period, probably, a trace of phosphatase acted slowly on the phosphate and gradually dephosphorylated the conjugate, resulting in the generation of anti-Ptyr antibody in mice sera however the screening procedure would select anti-BSA antibodies.

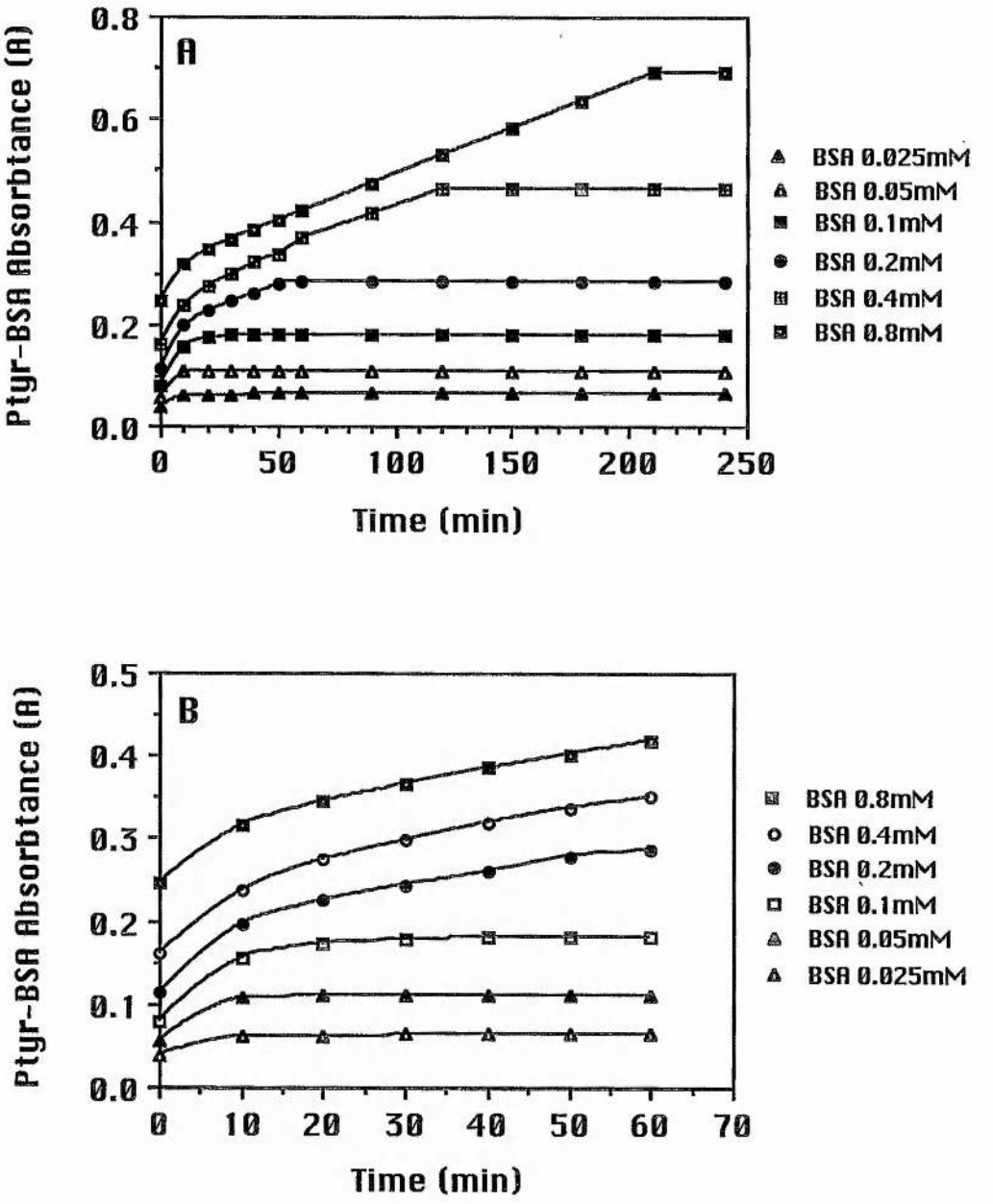


Fig.I-14. The time of thiol-disulphide exchange reaction determined by measuring the absorbance of the pyridine-2-thione released from the exchange reaction at λ_{max}=343nm. A. The 4 hours of the reaction; B. The first hour of the reaction.

2. MAb binding and DNA binding of DBP

To determine which part of DBP was recognized by MAb 12B8, purified viral early DBP (viral DNA replication was blocked by hydroxyurea during infection), late DBP (viral DNA replication was not blocked during infection) and baculo-viral DBP were digested with different amount of chymotrypsin and trypsin. The proteolysed DBP fragments were analysed by western blotting with rabbit polyclonal antibody (PAb) and MAb to DBP. No difference was found in Ag (antigen)-Ab (antibody) interaction pattern in western blotting between PAb & MAb (Fig.1-8), early DBP & late DBP (data not shown) and viral DBP & baculo-viral DBP (data not shown). Probably these three species of DBP contain the same proteolytic cleavage sites in a purified form and present a major antigenic site in a denatured form. DBP may contain only a few linear antigenic epitopes and the major one was recognized by MAb 12B8 since PAb apparently did not detect more fragments of digested protein as one would expect. It is possible (not necessary) that this major linear epitope may be located at C-terminal since after intensive treatment with proteases (the weight ratio of DBP to protease was up to 2.5:1), one major band appeared in western blotting with an apparent size of about 39K (Fig.1-8, Lanes 3 & 4, 7 & 8) and this has been shown by previous results to be derived from C-terminal.

It is of interest to know whether binding of DBP to this MAb (12B8) affects the ability of DBP to bind to DNA and vice versa. Thus, MAb binding and DNA binding experiments were carried out and performed first employing a dot blotting

assay and then using a gel retardation assay. In a dot blotting assay, DBP was absorbed to a nitrocellulose filter paper and the paper was blocked with 10% Marvel/PBS followed by incubation with (1) ^{32}P-labelled ssDNA (control) (2) MAb then radioactive ssDNA (3) labelled ssDNA then MAb. DNA binding can be identified by autoradiography and MAb binding can be identified by immunostaining with peroxidase linked anti-mouse IgG. Surprisingly, no radioactivity was detected in any of the blots, suggesting that DNA did not bind to DBP under these conditions. When DNA was absorbed to nitrocellulose filter paper and the paper was blocked with Marvel and then incubated with DBP followed by an antibody to DBP, a positive result was obtained (data not shown), indicating that DBP had bound to DNA. Probably when DBP attached to the filter paper, the availability of its conformation was reduced. For MAb binding, it may require mainly an exposed antigenic epitope while for DNA binding, it requires not only the primary structure but also the environmental conformation and possibly the inducibility of the conformation change of the protein to maintain the binding. Compared with aqueous media, when DBP was absorbed to a solid matrix (nitrocellulose filter paper), its inducibility in conformation change was reduced and this probably limited its ability to bind to DNA. In a gel retardation assay (Fig.I-15, Part II Methods 7), 0.5μg of purified DBP was either incubated (0°C, 30 min) with MAbs 12B8 (purified form at 1mg/ml, Lanes 3-6 corresponding to dilutions of 1:8, 1:4, 1:2 & 1:1 respectively) and A1 (ascitic fluid, Lanes 7-9 corresponding to dilutions of 1:8, 1:4 & 1:2 respectively) prior to reaction (0°C, 15 min) with

ssDNA (1μl, ^{32}P labelled 18bp oligonucleotide at 100 counts/μl/min) or incubated (0°C, 15 min) with ssDNA prior to reaction (0°C, 30 min) with MAbs 12B8 (Lanes 10-13) and A1 (Lanes 14-16). From figure 1-15, it is interesting to see: (1). When DBP was incubated with ssDNA only, there were two bands of DBP-DNA complex with different mobilities (Lane 2). (2). When DBP was incubated with MAb 12B8, which recognizes a linear epitope of DBP (see this part, results 3), prior to reaction with DNA, the top band was further retarded as the amount of 12B8 increased while the bottom band remained the same (Lanes 3-6). When DBP was incubated with DNA prior to reaction with 12B8, the same result (interaction pattern) was obtained (Lanes 10-13). (3). When DBP was incubated with MAb A1, which recognizes a conformational (or discontinuous) epitope of DBP (Reich et al, 1983), prior to reaction with DNA, both the top and bottom bands were retarded further as the amount of A1 increased (Lanes 7-9). When DBP was incubated with DNA prior to reaction with A1, the top band was further redarded and the bottom band remained the same (Lanes 14-16). To confirm these results, the same experiment was repeated using a reduced amount of DBP (0.1μg instead of 0.5μg) and increased amounts of MAb (12.5 fold excess instead of 5 fold) and ssDNA probe (1.5μl instead of 1μl), and the same results were obtained (Fig.1-16). When DBP reacted with ssDNA at low concentrations (<0.1μg/20μl reaction volume), probably it involved mainly a protein-DNA interaction and DBP may bind to DNA as monomers, resulting in one retarded band in the gel with higher mobility (Fig.1-16, Lane3). When the amount of DBP

Fig.I-15. Gel retardation assay of MAb binding & DNA binding of DBP. Lane1: ssDNA (1μl); Lane2: DBP (0.5μg)/ssDNA (control); Lanes3-6: DBP/12B8 (1:8, 1:4, 1:2 & 1:1)/ssDNA; Lanes7-9: DBP/A1 (1:8, 1:4 & 1:2)/ssDNA; Lanes10-13: DBP/ssDNA/12B8 (1:8, 1:4, 1:2 & 1:1); Lanes14-16: DBP/ssDNA/A1 (1:8, 1:4 & 1:2). The molar ratio of DBP:MAb used here was 1:1.25, 1:2.5, 1:5 and 1:10 corresponding to dilutions of MAb of 1:8, 1:4, 1:2 and 1:1 for 12B8.

increased in the same reaction volume, apart from the protein-DNA interaction, protein-protein interaction may occur and DBP may oligomerize and bind to DNA as oligomers

e.g. dimers, resulting in two retarded bands in the gel with different mobilities (Fig.I-16, Lanes 2 & 1; Fig.I-15, Lane2). When MAb bound to DBP, it did not affect the availability of DBP for DNA binding since there was a shift upon binding of DNA to the DBP-MAb complex (Fig.I-15, Lanes 3-9; Fig.I-16, Lanes 4 & 5 and 8 & 9), but the binding of DBP to DNA affected the binding of DBP to MAbs (Fig.I-15, Lanes 10-16; Fig.I-16, 6 & 7 and 10 & 11) and the stability of the DBP-MAb complex

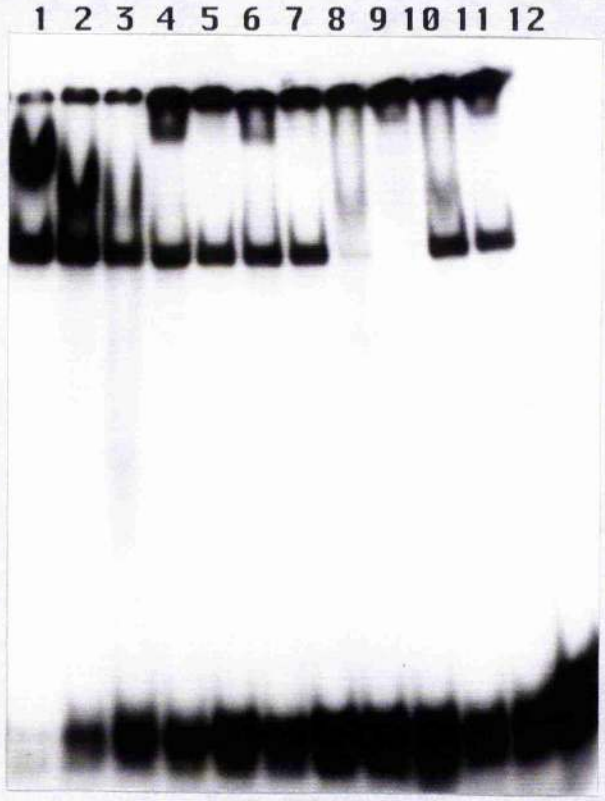


Fig.I-16. Gel retardation assay of MAb binding & DNA binding of DBP. Lane1: 0.5μg DBP/ssDNA (1.5μl); Lane2: 0.2μg DBP/ssDNA; Lane3: 0.1μg DBP/ssDNA (control); Lanes 4 & 5: DBP/12B8 (1:4 & 1:1)/ssDNA; Lanes 6 & 7: DBP/ssDNA/12B8 (1:4 & 1:1); Lanes 8 & 9: DBP/A1 (1:4 & 1:1)/ssDNA; Lanes 10 & 11: DBP/ssDNA/A1 (1:4 & 1:1); Lane12: ssDNA/12B8 (1:1) + A1 (1:1). The molar ratio of DBP:MAb used here was 1:6.25 and 1:25 corresponding to dilutions of MAb of 1:4 and 1:1 for 12B8.

(Fig.I-15, Lanes 3-6; Fig.I-16, Lanes 4 & 5). Binding of DBP to DNA apparently induces a conformational change in DBP as well as DNA and this change probably in turn stabilizes the DBP-DNA complex. When DBP was incubated with DNA prior to the addition of MAb, the conformation change induced in the protein upon binding of DNA may alter its discontinuous epitopes or shelter its linear epitopes, resulting in the failure of these epitopes to be recognized by MAbs such as 12B8 and A1 (lower shifted bands of Fig.I-15, Lanes 10-16; Fig.I-16, Lanes 6 & 7 and 10 & 11). In the case of oligomers of DBP (when protein-protein interaction was involved), some DBP molecules may be not in direct contact with DNA or their conformation had not been changed much, retaining the availability of the epitopes for MAb binding (higher shifted bands of Fig.I-15, Lanes 10-16; Fig.I-16, Lanes 6 & 7 and 10 & 11). In the case of DBP-MAb complex, when MAb binding did not affect DNA binding e.g. DBP-12B8 and DBP-A1, the induced conformational change in DBP upon binding to DNA may result in a disassociation of DBP from MAb (lower shifted bands of Fig.I-15, Lanes 3-6; Fig.I-16, Lanes 4 & 5).

Part II Tyrosine phosphorylation of DBP

Before anti-Ptyr antibodies had been established, phosphotyrosine-containing protein was detected by alkali treatment of ^{32}P labeled samples separated in a SDS gel prior to autoradiography (Cooper et al, 1983), or by base hydrolysis of the samples followed by phosphoamino acid analysis (Martensen and Levine, 1983), The former technique assumed that the phosphate ester linkage to tyrosine is stable to high pH. However not all phosphotyrosyl residues are resistant to high pH and phosphothreonine in some situations is also stable to high pH. Now anti-Ptyr antibodies are most widely used in detecting phosphotyrosine-containing proteins in immunoassay (Yin and Wang, 1988) such as western blotting and immunoprecipitation. Proteins containing phosphotyrosine thus identified can then be purified by immunoaffinity chromatography (Pang et al, 1985).

Using synthetic peptides, the activity of protein tyrosine kinases can be measured by incubating the protein with the peptide in the presence of (γ-^{32}P)ATP (radioactive assay) or ATP (nonradioactive assay). Tyrosyl-phosphorylated peptide can then be counted for ^{32}P-labeling by adherance to phosphocellulose paper or identified by anti-ptyr antibodies in the case of nonradioactive labeling.

Peptide RR-SRC (Bowen et al, 1991) and poly (Glu.Na, Tyr) 4:1 (Rijksen et al, 1989; Lazaro et al, 1991) are artificial substrates used in tyrosine kinase assay. RR-SRC is designed for a filter assay with two arginines attached to the amino

terminus. Poly (Glu.Na, Tyr) 4:1 can be linked to a ELISA plate, facilitating a nonradioactive assay for tyrosine kinase.

Methods:

1. Preparation of A431 cell extracts containing EGFR (Yarden and Schlessinger, 1987)

Membrane extracts A431 human epidermoid carcinoma cells were grown in G-MEM/10% NCS in 8cm dishes to confluency, washed twice with PBS, scraped off and pelleted in PBS at 600g for 10 min (4°C, benchcentrifuge). The cell pellet was resuspended in 10 volumes of hypotonic buffer (10mM Hepes, 1.5mM $MgCl_2$, 1mM PMSF and 1mM EGTA at pH 7.4) and incubated at 4°C for 10 min. The lysate was briefly homogenized by 3-5 strokes with a glass dounce homogenizer and centrifuged (600g, 10 min, 4°C). The pellet was discarded and the supernatant was further centrifuged at 24,000g for 30 min at 4°C. The resulting pellet was then resuspended in 20 volumes of HNEG buffer (20mM Hepes, 150mM NaCl, 1mM EGTA and 10% Glycerol at pH 7.4), homogenized (10 strokes) and loaded on 35% sucrose/PBS. After centrifugation at 24,000g for 30 min at 4°C, the interface fraction was collected, diluted 1:5 in 10mM Hepes (pH 7.4) and recentrifuged at 100,000g for 20 min at 4°C. The resulting pellet was resuspended in 20mM Hepes pH 7.4, aliquoted and stored at -70°C.

Solubilized extracts Confluent A431 cells were washed twice with HNEG (20mM Hepes/150mM NaCl/1mM EGTA/10% Glycerol, pH 7.4) and pelleted in HNEG. The cell pellet was stored at -70°C until use. The frozen cells were suspended in 1ml of

solubilization buffer (50mM Hepes, 150mM NaCl, 1% Triton X-100, 10% Glycerol, 1.5mM $MgCl_2$, 1mM EGTA and 1mM PMSF containing 0.15 trypsin inhibitor unit/ml of aprotinin and 10μg/ml of leupeptin at pH 7.5), homogenized (14 strokes) and clarified at 40,000g for 30 min at 4°C. The supernatant was aliquoted and stored at -70°C.

2. EGF stimulation of A431 cells and cell extracts

To A431 cells (in vivo stimulation), EGF was added at 100ng/ml to culture medium 0.5-1 hour prior to harvesting the cells; For A431 cell extracts (in vitro stimulation), EGF was added at 500ng/ml to the extracts in a binding buffer (50mM Tris-HCl/150mM NaCl, pH 7.5), and the mixture was incubated on ice for 10 min prior to be labelled with ^{32}P in vitro (i.e. in vitro stimulation of the extracts with EGF was followed immediately by in vitro phosphorylation of the same extracts).

3. In vitro phosphorylation

10μl of cell extracts was incubated with 2-5μCi of (γ-^{32}P)ATP in an in vitro phosphorylation buffer (25mM Tris-HCl, 10mM $MnCl_2$, 0.5mM EGTA, 50μM Na_3VO_4 and 20μM $ZnSO_4$ at pH 7.5) on ice for 30 min. Immediately after that, the reaction mix was immunoprecipitated with an antiserum to DBP or Ptyr. For western blotting, 250μM of ATP was used as phosphate donors instead of (γ-^{32}P)ATP. Immediately after phosphorylation, the mixtures were denatured in sample buffer (100°C, 3 min), separated by SDS PAGE and blotted to nitrocellulose filter paper, followed by probing with antisera.

4. Immunoprecipitation

^{32}P-labeled cell extracts were incubated with 5μl of antisera on ice for 2 hours. After which, 100μl of Staph A (fixed Staphylococcus aureus of Cowan A strain in a 10% w/v suspension) was added and the mixture was incubated for a further 30 min on ice. Immune complexes absorbed to Staph A were then isolated by centrifugation at 2500g for 3 min (bench microcentrifuge) and washed three times with washing buffer (20mM Tris-HCl/0.65M NaCl/1mM EDTA/0.5% NP40/10% Sucrose at pH 7.5). The proteins in the complexes were dissociated by boiling in sample buffer and analysed by SDS PAGE followed by autoradiography.

5. Protein tyrosine kinase assay

Radioactive filter assay (Bowen et al, 1991) 4μl (10μg) of cell extracts were incubated with 1μl (15μg) of RR-SRC (synthesised by Paul Talbot) and 1μCi of γ-ATP in a 20μl volume containing 30mM Hepes (pH7.4), 10mM $MgCl_2$, 0.1mM DTT, 25μg/ml BSA, 0.15% NP40, 70μM Na_3VO_4 and 60μM ATP on ice for 30 min. After which, 120μl of 5% (w/v) trichloroacetic acid (TCA) was added and the mixture was left on ice for 1 hour before centrifugation at 12,000g for 10 min. 20μl of the supernatant was spotted onto a phosphocellulose disc (cut from a Whatman p81 phosphocellulose paper). The discs were washed twice with 1% (v/v) acetic acid, twice with water, air dried, placed in 5-10ml of scintillation fluid and counted (TRI-CARB 300 Liquid Scintillation System, Canberra Packard).

Nonradioactive ELISA (Lazaro et al, 1991) A 96-well ELISA plate was treated with 1% Glutaraldehyde (Sigma, 25%

solution) and washed in PBS prior to use. Poly (Glu.Na, Tyr) 4:1 (Sigma) was absorbed to the pretreated plate at 10ng/ml in PBS overnight at 4°C. The plate was washed with PBS, blocked with 5% Marvel/PBS (37°C, 30 min), and 50µl of assay buffer (50mM Hepes, 150mM NaCl, 10mM $MgCl_2$, 5mM NaF, 50µM Na_3VO_4, 0.8mM EGTA, 0,2mM EDTA and 300µM ATP at pH 7.4) was added followed by 50µl of cell extract diluted in assay buffer containing no ATP. The phosphorylation reaction was carried out at 37°C for 30 min. After which, the plate was washed with PBS and anti-Ptyr antibody (diluted in 5% Marvel/PBS) was applied (37°C, 1 hour) followed by incubation with peroxidase-labeled secondary antibody (37°C, 30 min). Finally, phosphorylated tyrosine was visualized by color development.

6. Iodination of DBP (Ruyechan and Olson, 1992)

1ml of each purified DBP (early 300µg/ml, late 800µg/ml and baculo-viral 400µg/ml) was dialyzed against 0.1M sodium borate (pH 9.5) for 2h at 4°C. The resulting 2ml of protein solution was split into four 0.5ml fractions. Of which, three fractions were treated with 1.25µl, 2.5µl and 5µl of KI_3 (0.05M I_2 in 0.24M KI), and one fraction was treated with 5µl of H_2O (as a control). The reaction mixtures were incubated on ice for 20 min and the reaction was stopped by the addition of 15µl of 1M $NaHSO_4$. Iodinated and control protein fractions were then dialyzed against 10mM Tris-HCl (pH 7.5), 150mM KCl, 1mM EDTA, 0.1mM DTT, 20µg/ml PMSF and 50% Glycerol overnight at 4°C.

7. Gel shift DNA binding assay (Stuiver et al, 1992)

25-200ng of DBP (untreated or iodinated) was incubated with 1μl of ^{32}P labelled ssDNA oligonucleotide (18bp from Ad2 origin, 100 counts/min) in 25mM Hepes (pH 7.4), 100mM NaCl, 1mM DTT, 5mM $MgCl_2$, 0.01% (v/v) NP-40 and 0.1mg BSA/μl (binding buffer) in a total volume of 20μl on ice for 15min. Then, 5μl of 5x DNA sample buffer (50mM Tris-borate/1mM EDTA/0.02% Bromophenol blue/50% Glycerol at pH 8.3) was added and the reaction mix was loaded onto a 6% nondenatured acrylamide/bisacrylamide gel, which was 6% of acrylamide:N,N'methylene-bisacrylamide (44:0.8 w/w) in 50mM Tris-borate/0.1% $(NH_4)_2S_2O_8$/0.12% TEMED at pH 8.3. The electrophoresis was carried out at 200^v of constant voltage for 1-2.5h at 4°C. After that, the gel was fixed by DEAE paper, dried and exposed to Fuji X-ray film.

Results:

1. Tyrosyl-phosphorylation of DBP during Ad2 infection

It was suggested that DBP is tyrosyl-phosphorylated early after infection. In vivo ^{32}P labelling of Ad2 infected Hela cells at different post-infection times showed that the degree of labelling of the DBP increased up to relatively late times while the proportion of alkali-resistant phosphorylation was greatest at the early infection times (Russell et al, 1989). To confirm this, DBP phosphorylation was carried out in an in vitro system and phosphorylation of DBP was determined by immunoprecipitation using rabbit polyclonal antibodies against DBP and Ptyr as

follows: Hela monolayer cells were infected with Ad2 at 20 p.f.u/cell and extracted in an in vitro phosphorylation buffer at post-infection (p.i) of 12 hours (h), 18h, 24h, 30h and 36h. In vitro phosphorylation was carried out by incubating 10μl of each infected cell extract with 2.5 μCi of (γ-^{32}P)ATP on ice for 30 min. ^{32}P labelled extracts were then parallelly precipitated with anti-DBP and anti-Ptyr antibodies (Fig.II-1). Figure II-1 shows that DBP was phosphorylated during the time course of Ad2 infection (Lanes 1-5) but phosphotyrosine was not detected in phosphorylated DBP during infection (Lanes 6-10), suggesting that tyrosine phosphorylation of DBP may not be represented in an in vitro system.

In an immunofluorescence assay, a time course of Ad2 infected Hela cells were fixed, penetrated and probed with antibodies against DBP and Ptyr followed by immunostain with fluorescence labelled secondary antibody. DBP appeared in some infected cells as early as 6 hours but phosphotyrosine was not detected during the course of 3-48h p.i (data not provided), suggesting that there is no detectable increase in phosphotyrosine level in Hela cells during Ad2 infection and if DBP is phosphorylated early after infection, the signal of Ptyr would be too low to be detected in this assay.

2. In vitro tyrosine phosphorylation of purified DBP

Phosphotyrosine of DBP was not detected in crude Ad2 infected cell extracts after in vitro phosphorylation. How about purified DBP? Can it be phosphorylated at tyrosine in vitro? To ascertain whether DBP is a substrate of protein tyrosine kinases, in vitro phosphorylation of purified DBP was

performed using Ad2 infected and mock-infected Hela cell extracts and EGF stimulated A431 cell extracts as different sources of tyrosine kinases. Uninfected and Ad2 infected Hela cell extracts served as crude cellular and viral kinases while EGF stimulated A431 cell extracts presented mainly receptor

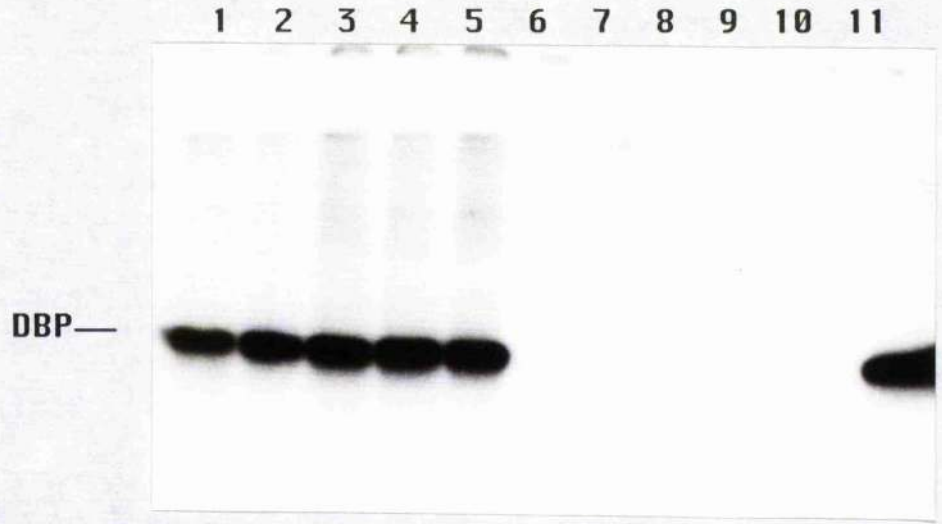


Fig.II-1. Autoradiography of a time course of Ad2 infected Hela cell extracts immunoprecipitated with antibodies against DBP and Ptyr after the extracts have been labelled with ^{32}P in vitro. The extracts in lanes 1-5 and 11 were immunoprecipitated with an anti-DBP antibody and the extracts in lanes 6-10 were immunoprecipitated with an anti-Ptyr antibody. Lanes 1&6: 12h p.i; Lanes 2&7: 18h p.i; Lanes 3&8: 24h p.i; Lanes 4&9: 30h p.i; Lanes 5&10: 36h p.i; Lane 11: purified DBP was added to 24h p.i extracts.

(EGFR) kinase since A431 cells contain very high level of receptors specifically to EGF. These receptors possess a tyrosine kinase activity and this activity is increased (several fold) upon binding to (stimulating with) EGF. EGFR itself is autophosphorylated at several sites of tyrosine residues,

providing not only a tyrosine kinase but also a substrate for tyrosine kinase. Phosphorylated EGFR has an apparent molecular weight of about 175K in SDS gel. After labelling of A431 cell extracts and Ad2 infected or mock-infected Hela cell extracts with ^{32}P, the reaction mixes were parallelly immunoprecipitated with anti-DBP and anti-Ptyr antibodies, and separated by SDS PAGE followed by autoradiograph(Fig.II-2). From figure II-2, it can be seen that phosphorylated DBP only presented in the samples immunoprecipitated with anti-DBP antibody (Lanes 3, 6, 8, 11 and 13) but not with anti-Ptyr antibody (Lanes 4, 7, 9, 12 and 14) while tyrosine phosphorylated EGFR was immunoprecipitated with anti-Ptyr antibody as expected (Lanes 12 and 14). This suggests that although purified DBP can be phosphorylated in vitro by both cellular (Lanes 3, 11, and 13) and viral (Lane 8) kinases it may not be a substrate of tyrosine kinases since it was apparently not tyrosyl-phosphorylated by Hela cellular kinases (Lane 4), viral kinases (Lanes 7 and 9) and crude EGFR (Lanes 12 and 14).

A similar experiment was carried out by western blotting and the same results were obtained (Fig.II-3; Fig.II-4). In western blotting, purified U-DBP (wild type viral DBP) and B-DBP (DBP expressed in Baculovirus) were incubated with Ad2 infected Hela cell extracts and EGF stimulated A431 cell extracts in the presence of ATP. After in vitro phosphorylation, the samples were separated by SDS PAGE, blotted to nitrocellulose filter paper and probed with both anti-DBP (A) and anti-Ptyr (B) antibodies. Figures II-3 and II-4

show that EGFR (in A431 cell extracts) was tyrosyl-autophosphorylated (Fig.II-3.B. Lanes 5 & 8; Fig.II-4.B. Lanes

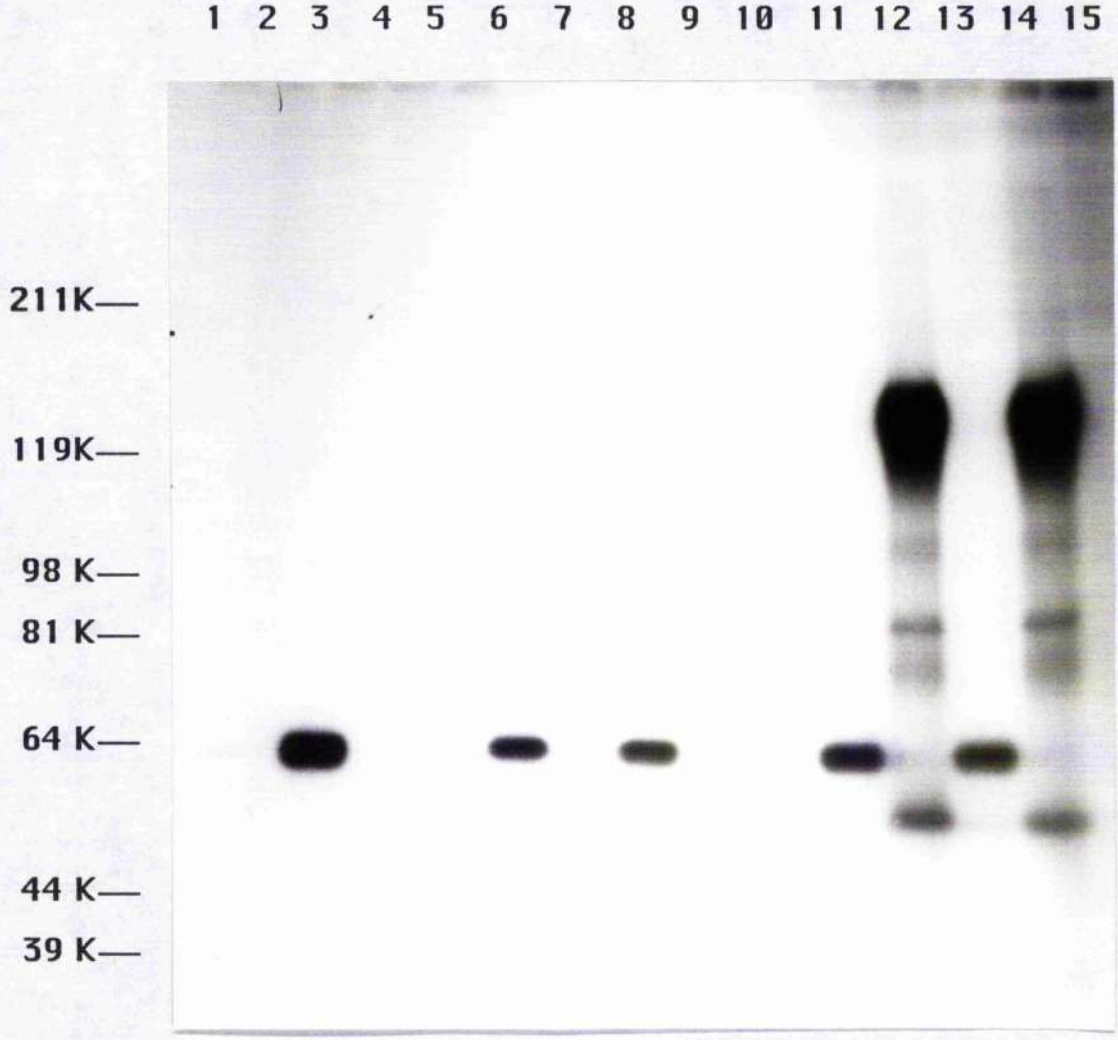


Fig.II-2. An autoradiography of in vitro phosphorylation of purified DBP immunoprecipitated with anti-DBP and anti-Ptyr antibodies. Lane1 (2): uninfectedHela cell extracts immunoprecipitated with anti-DBP(anti-Ptyr) antibody; Lane 3 (4): uninfected Hela cell extracts plus purified DBP immunoprecipitated with anti-DBP (anti-Ptyr) antibody; Lane 5: the same sample as lanes 3 and 4 but immunoprecipitated with a control rabbit antiserum (anti-SV5); Lane 6 (7): infected Hela cell extracts immunoprecipitated with anti-DBP (anti-Ptyr) antibody; Lane 8 (9): infected Hela cell extracts plus purified DBP immunoprecipitated with anti-DBP (anti-Ptyr) antibody; Lane 10: control antiserum to SV5; Lane 11 (12): EGF stimulated A431 cell membrane extracts plus purified DBP immunoprecipitated with anti-DBP (anti-Ptyr); Lane 13 (14):

EGF stimulated A431 cell solubilized extracts plus purified DBP immunoprecipitated with anti-DBP (anti-Ptyr); Lane 15: control antiserum to SV5.

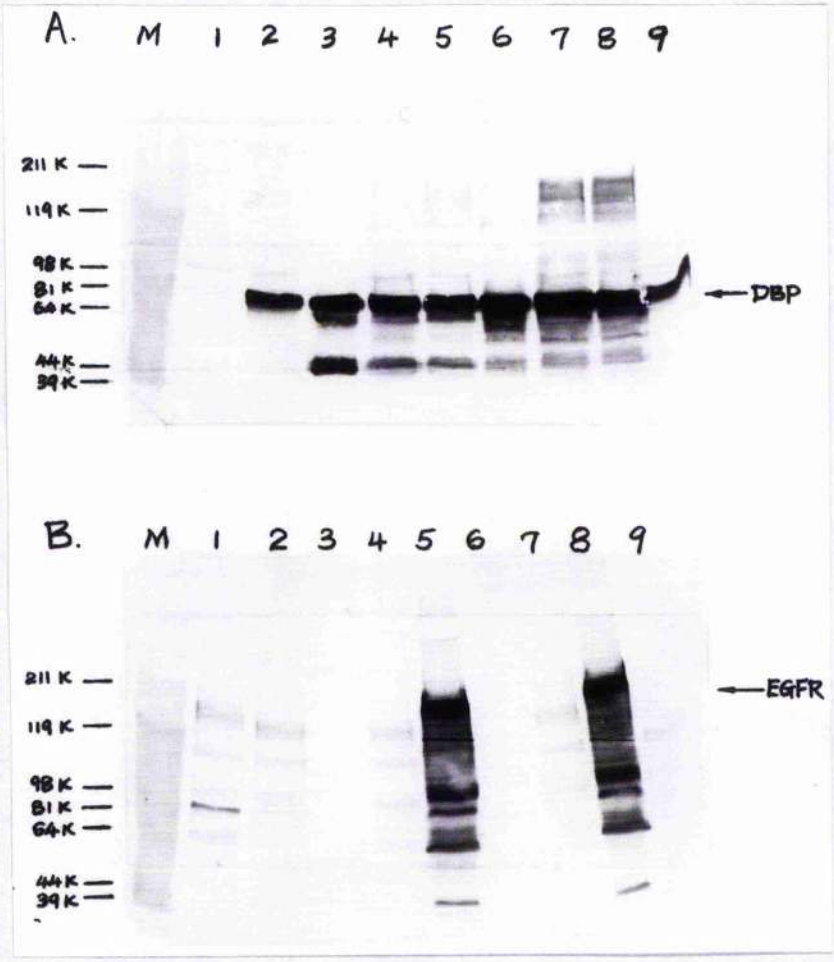


Fig.II-3. Western blotting of purified DBP after in vitro phosphorylation. A: The blot was probed with an anti-DBP antibody. B: The blot was probed with an anti-Ptyr antibody. Lane 1: uninfected Hela cell extracts; Lane 2: infected Hela cell extracts; Lane 3 (6): purified U-DBP (B-DBP); Lane 4 (7): infected Hela cell extracts plus purified DBP (B- DBP); Lane 5 (8): EGF stimulated A431 cell solubilized extracts plus purified U-DBP (B-DBP).

1 & 2) in this assay system, but both purified U-DBP (Fig.II-3. B. Lanes 4 & 5; Fig.II-4.B. Lane 2) and B-DBP (Fig.II-3.B. Lanes

7 & 8) were not, suggesting that purified DBP may not be a substrate for tyrosine kinase in vitro. It was also shown in Fig.II-3.B that purified DBP does not contain phosphotyrosine

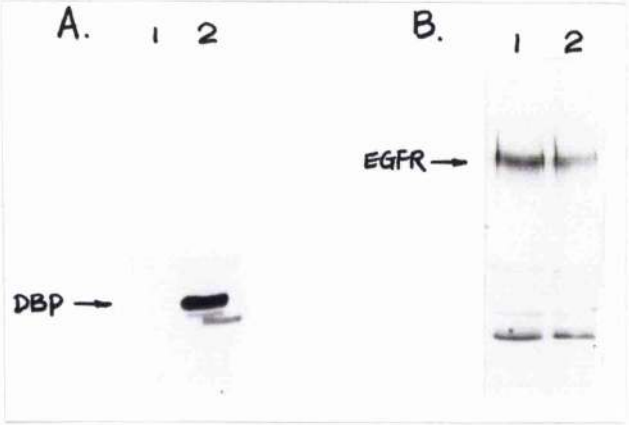


Fig.II-4. Western blotting of purified DBP after in vitro phosphorylation by EGF stimulated A431 cell membrane extracts. A: probed with anti-DBP antibody; B: probed with anti-Ptyr andtibody. Lane 1: membrane extracts; Lane 2: membrane extract plus purified DBP.

(Lanes 3 & 6), suggesting that if DBP is tyrosyl-phosphorylated in vivo, it may be dephosphorylated at tyrosine during the extraction and purification process.

3. In vitro tyrosine phosphorylation of peptide DBP195

DBP was shown (Russell et al, 1989) to be tyrosyl-phosphorylated by in vivo labelling the DBP with ^{32}P and the putative tyrosine phosphorylation site was suggested to be at Try_{195}. By in vitro (Russell et al, 1989) labelling the DBP with ^{32}P, DBP was not shown to be phosphorylated at tyrosine (Fig.II-1, 2, 3, and 4). To find out whether the primary sequence of DBP that contains the putative tyrosine phosphorylation site (Tyr_{195}) is

phosphorylatable, a peptide DBPPT195 (RRALMDKYHVDNDL) was

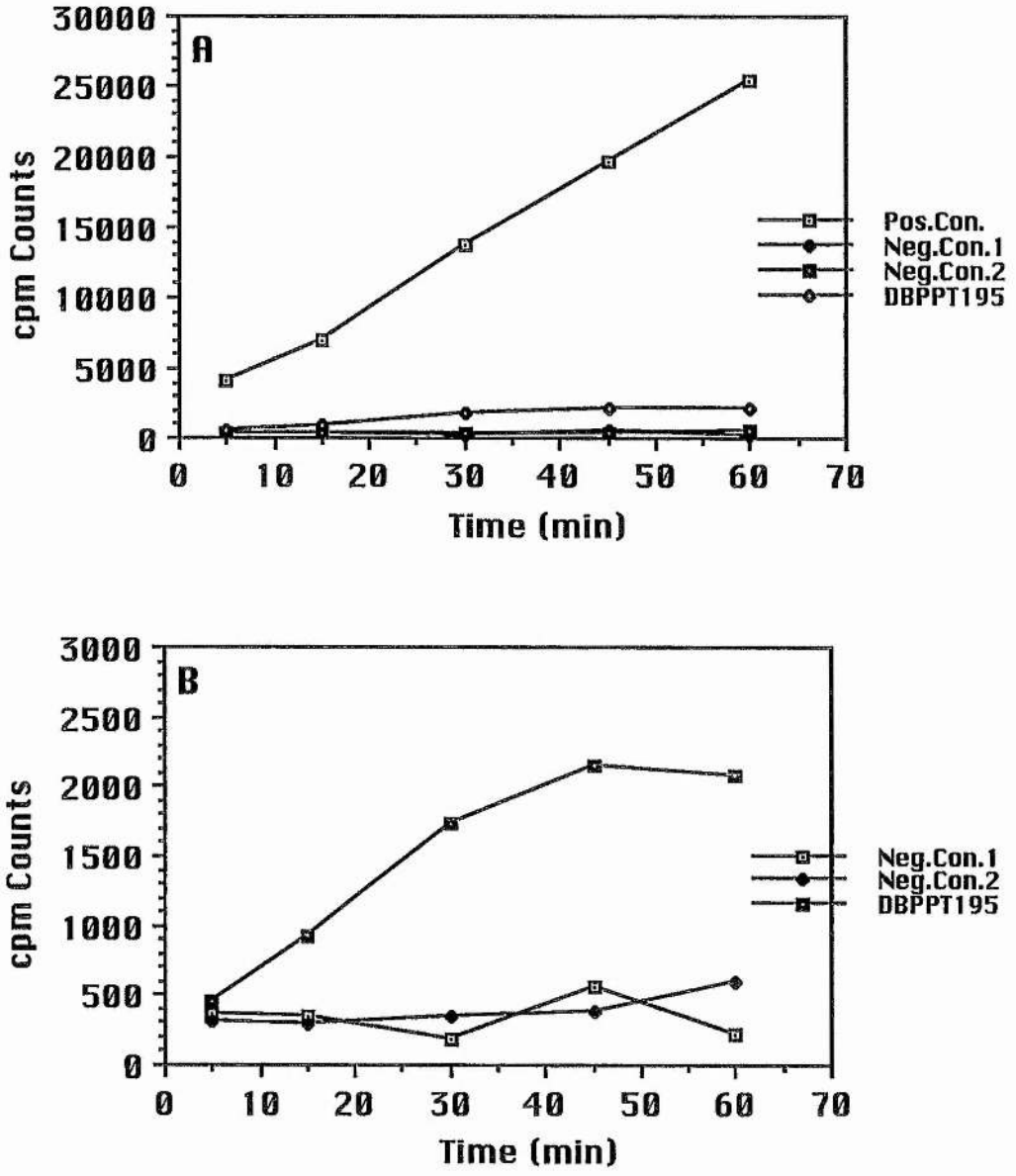


Fig.II-5. A time course of in vitro phosphorylation of peptide DBP PT195. Pos.Con: positive control of EGF stimulated A431 cell solubilized extracts with peptide RR-SRC; Neg.Con.1: negative control of EGF stimulated A431 cell solubilized extracts with peptide containing no tyrosine (ETGGVHWS); Neg.Con.2: negative control of EGF stimulated A431 cell solubilized extracts with no peptide; DBPPT195: EGF stimulated A431 cell solubilized extracts with peptide DBPPT195. A. Presentation of cpm counts of all the samples after spotting onto nitrocellulose and washing with PBS. B. To provide a

better view of DBP PT195 phosphorylation, graph of Pos.Con. was withdrawn from the figure.

synthesized from the sequence of Ad2 DBP (aa188-aa201) and an in vitro phosphorylation of this peptide was performed using A431 cell solubilized extracts as crude tyrosine kinases (containing mainly EGFR receptor kinase). Fig.II-5 shows the result that it was phosphorylated although the extent of its phosphorylation was much lower than the peptide RR-SRC (Fig.II-5). This could be because RR-SRC probably binds to phosphocellulose paper better than DBPPT195 since it contains two arginine residues at the end while DBPPT195 does not.

4. Tyrosine kinase assay on Ad2 infected Hela cells

In an in vitro system, DBP was not shown to be tyrosine-phosphorylated in crude cell extracts and in a purified form as described above. But a peptide selected from the DBP sequence that contains a putative tyrosine phosphorylation site was shown to be phosphorylated in vitro by a crude receptor kinase (Fig.II-5). This implicates a potential that DBP may be tyrosyl-phosphorylated in vivo when this site (Try_{195}) is accessible to tyrosine kinases under certain circumstance. To find out whether there is an increase in tyrosine kinase activity in Ad2 infected Hela cells, an in vitro phosphorylation was performed using synthetic peptide RR-SRC. RR-SRC (RRLIGDAEYAARG) an artificial substrate for several tyrosine kinases, generated from a tyrosine phosphorylation site in $pp60^{src}$ (Casnellie et al, 1982). To test whether tyrosine kinase activity increases in Ad2 infected Hela cells, peptide RR-SRC was incubated with Ad2 infected and mock-infected

Hela cell extracts in an in vitro phosphorylation buffer in the presence of γ^{32}P-ATP. As a comparison, RR-SRC was also incubated with EGF stimulated and mock-stimulated A431 cell membrane extracts. After ^{32}P labeling, the proteins in the reaction mix were removed by acid precipitation and tyrosyl-phosphorylated peptide (RR-SRC) was adhered to a phosphocellulose paper and counted for radioactivity in scintillation fluid (Fig.II-6). From figure II-6, it can be seen clearly that tyrosine kinase activity was not increased in Ad2 infected Hela cells at both early and late times. In fact it appeared that Ad2 infection slightly down-regulated cellular tyrosine kinase activity (Fig.II-6, B). It has been reported (Carlin et al, 1989; Verheijden et al, 1992) that group C human adenoviruses down-regulate EGFR of a variety of cell types early after infection. Hela cells contain very low level of EGFR (Hunter and Cooper, 1981), and whether this little decrease in activity could be attribute to EGFR or not is not clear.

A similar experiment was carried out by ELISA using an artificial substrate of poly (Glu.Na, Tyr) 4:1 and a similar result was obtained (data not provided): there was apparently no increase in activity of tyrosine kinase in Ad2 infected Hela cell extracts.

Since peptide DBPPT195 can be phosphorylated by EGFR (Fig.II-5) and its sequence was selected from DBP containing a putative tyrosine phosphorylation site, will it serve as a better substrate for viral tyrosine kinase? The same kinase assay as with peptide RR-SRC was repeated using peptide DBPPT195 and no tyrosine kinase activity was found in both uninfected and Ad2 infected Hela cells (data not shown).

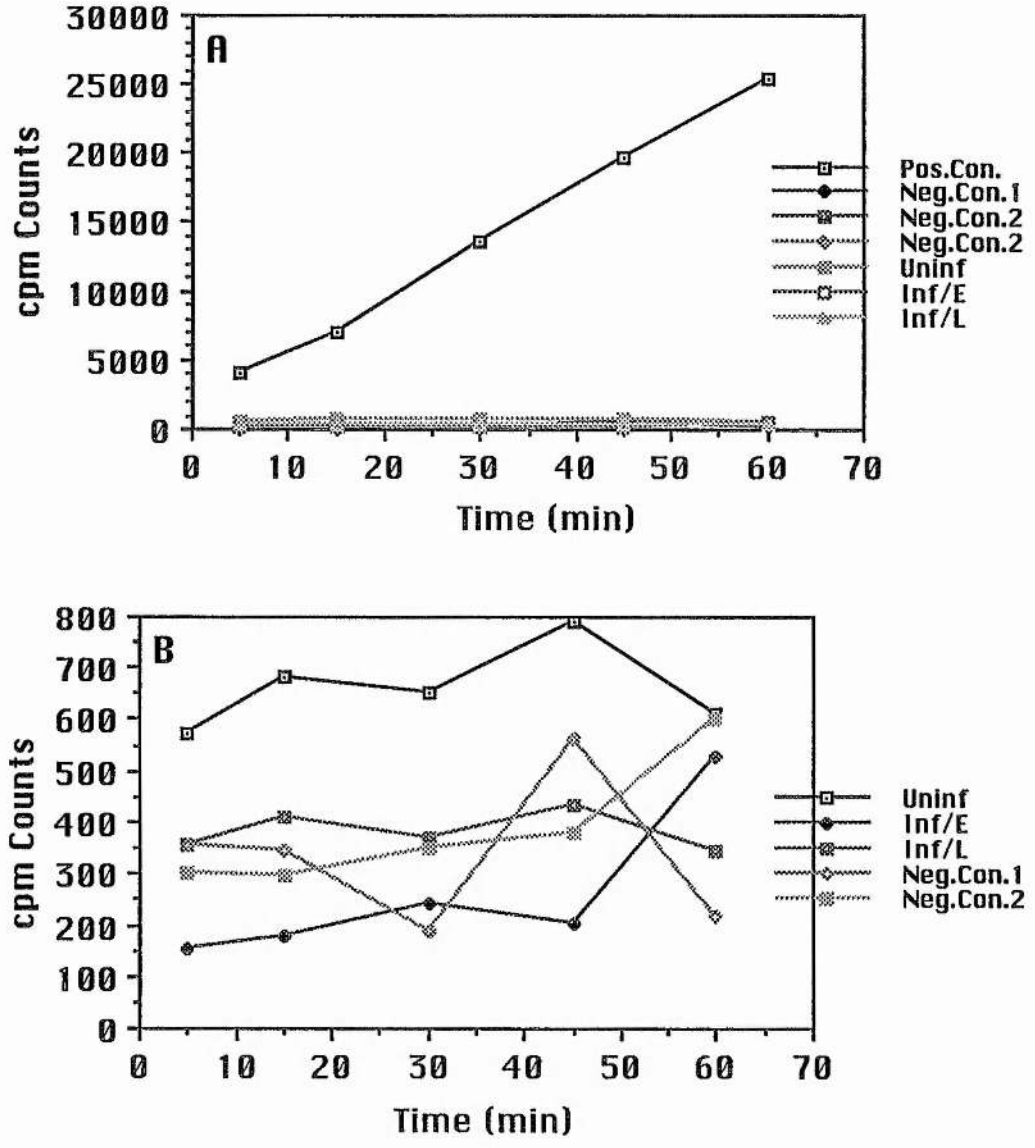


Fig.II-6. A time course of ^{32}P-incorporation in RR-SRC peptide by Ad2 infected and mock-infected Hela cell extracts. Pos.Con: positive control of EGF stimulated A431 cell solubilized extracts; Neg.Con.1: negative control of EGF stimulated A431 cell solubilized extracts with a peptide containing no tyrosine; Neg.Con.2: negative control of EGF stimulated A431 cell solubilized extracts with no peptide; Uninf: uninfected Hela cell extracts; Inf/E: Ad2 infected/hydroxyurea blocked Hela cell extracts; Inf/L: Ad2 infected unblocked Hela cell extracts. A. Presentation of cpm counts of all the samples. B. To make a better comparison, graph Pos.Con. was removed from Fig.A.

5. EGF effect on tyrosine kinase activity of Ad2 infected Hela cells

It is known that EGF stimulates tyrosine kinase activity of EGFR significantly. To test whether it affects tyrosine phosphorylation of Ad2 infected and mock-infected Hela cells (would phosphotyrosine signal be increased by an increased tyrosine kinase activity if DBP is tyrosine-phosphorylated but the signal is too low to be detected under the conditions mentioned above?). Ad2 infected and mock-infected Hela cells were incubated with 100ng/ml EGF in culture medium for 1 hour at 37°C (in vivo stimulation) prior to being extracted. After extraction, Hela cell extracts were separated by SDS PAGE and probed with anti-DBP and anti-Ptyr antibodies. It appeared (Fig.II-7) that in both uninfected and Ad2 infected Hela cells there was no difference in tyrosine phosphorylation pattern between EGF stimulated (Lanes 2 and 4) and mock-stimulated (Lanes 1 and 3) cells. This was repeated (data not shown) with similar results when cells were stimulated with EGF in vitro (i.e. cell extracts were incubated with EGF prior to phosphorylation). From these results, it suggests that EGF does not affect tyrosine phosphorylation of uninfected and Ad2 infected Hela cells, and the phosphotyrosine signal in DBP was not increased (presuming it was present) by stimulating the infected cells with EGF in vivo and in vitro.

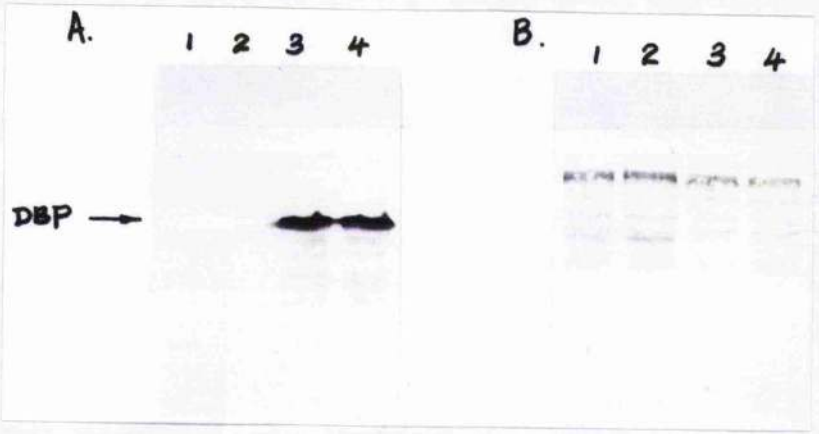


Fig.II-7. Western blotting on Ad2 infected and mock-infected Hela cells. A: probed with an anti-DBP antibody; B: probed with an anti-Ptyr antibody. Lane1: uninfected extract; Lane 2: in vivo EGF stimulated uninfected extracts; Lane 3: infected extract; Lane 4: in vivo EGF stimulated infected extracts.

Discussion:

1. Inability to detect Ptyr in DBP

Using anti-Ptyr polyclonal antibody, DBP was not detected to be phosphorylated at tyrosines in an immunofluorescence assay and in an in vitro phosphorylation system by immunoprecipitation and western blotting. Probably phosphotyrosine level is too low to be detected and in this regard immunoprecipitation and western blotting methods are not sensitive enough. Probably tyrosyl-phosphorylation turns on and off rapidly. It was reported (Kamps and Sefton, 1988) that phosphorylated tyrosine residues can be dephosphorylated during immunoprecipitation because of the presence of active phosphatases under this condition. Probably in an immunofluorescence assay, some

phosphotyrosine residues (on the protein) are sterically or conformationally hindered from binding to antibody, or perhaps that vicinal charges on the protein interfere with antibody binding (Frackelton er al, 1983); in this regard the use of a polyclonal antibody may be better than a monoclonal antibody. Probably in a western blotting assay, after SDS-PAGE and transfer to nitrocellulose filter paper, the accessibility of an antibody to phosphotyrosine would be changed (Linder and Burr, 1988). Apart from all these possibilities, an important factor could be that an in vitro system is not equivalent to an in vivo system and cannot represent the actual situation in vivo (Fujita-Yamaguchi et al, 1989). It is also possible that DBP might be not phosphorylated in vitro.

2.Tyrosyl-phosphorylation of DBP——does it take place?

It is not surprising that purified DBP failed to be phosphorylated at tyrosine by crude EGFR extract in vitro while a peptide selected from the DBP sequence containing the putative tyrosine phosphorylation site was shown to be phosphorylated. When a peptide contains a tyrosine which is surrounded by acidic residues, it is likely that the tyrosine would be phosphorylated by a tyrosine kinase. This is not necessary true in a protein, since the accessibility of the target amino acid to the kinase is governed by secondary or tertiary structure of the protein. In this regard, it is possible that DBP could be phosphorylated in vivo but may not in vitro and vice versa. In an in vivo situation, the conformation of DBP could be changed as the state of infection changes,

allowing the phosphorylation site of tyrosine to be exhibited or sheltered. Presuming DBP is phosphorylated in vivo (in some stage e.g. early after infection) as suggested in previous study, what possible roles would it play?

The involvement of tyrosine side chains in ssDNA binding of prokaryotic single-stranded DNA-binding protein has been demonstrated by the study of T4 bacteriophage gene 32 protein (Karpel, 1990), a non-sequence specific ssDNA binding protein which has served as a model of this class of protein and is well characterized. There are eight tyrosines in T4 gene 32 protein. Two of them have a structural function (stabilize the tertiary structure of the protein) while the remaining six are shown to be involved in ssDNA binding (each contrubutes to the overall affinity of protein for DNA). It is proposed that a discrete group of tyrosine residues may form regularly spaced hydrophobic pockets (a "ladder-like" binding groove) into which the heterocyclic rings of the oligonucleotide bind. There are six tyrosines in the C-terminal of DBP (for Ad2, Ad4 & Ad5) and three of them (corresponding to Try195, Try380 & Try455) are highly conserved among all serotypes (Fig.II-8). From figure II-8, it appears that all the tyrosines in C-terminal are distributed in a similar way at discrete sites. Are these tyrosines involved in DNA binding of DBP? Would it be possible that tyrosine phosphorylation of DBP indirectly regulates the overall affinity of the protein to DNA by altering the local hydrophobic state of the "binding groove" of the protein?

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Ad2   174  SLPI VSAWEKGMEAARALMDKYHVDNDLKANFKLLPDQVEALAAVCKTWLNEEHRGLQ
Ad4   153  SLPI VSAWEKGMEI MNMLMERYRVESDLKSNFQLMPEQGEVYR RICH LYI NEEHRGIP
Ad5   174  SVPI VSAWEKGMEAARALMDKYHVDNDLKANFKLLPDQVEALAAVCKTWLNEEHRGLQ
Ad7   159  SLPI VSAWEKGME VMAVLMERYRLDNDLRTSFKLMPEQHEQYKRI CHQYVNEEHRGIP
Ad12  129  SQPLVSAWEKGMEAMAMLMEKYHVDHDERATFRFLPDQGSVYKKICTTWLNEEKRGLQ
Ad40  120  AEPLVSAWEKGMDLM I KLMEKYHVEAEEKNGFKFLPEQSNVYRKICQTWLNEEHRGLP
Ad41  121  VDPLVSAWEKGMELM NVLMEKYHVENDEKTAFKFLPEQNAVYRKICQTWLNEERRGLS

Ad2   232  LTFTSNKTFVTMMGRFLQAYLQSFAEVTYKH HEPTGCALWLHRCAEIEGELKCLHGSI
Ad4   211  LTFTSNKTLTTMMGRFLQGFVHAHSQIAHKNWESTGCALWLHGCTEVEGKLRCLHGTT
Ad5   232  LTFTSNKTFVTMMGRFLQAYLQSFAEVTYKH HEPTGCALWLHRCAEIEGELKCLHGSI
Ad7   217  LTFSSMKTLTAMMGRFMQGLVHSYSEIAHNNWECTGCALWAHGCTDYEGKVKCLHGTI
Ad12  187  LTFSSQKTFQELMGRFLQGYMQAYAGVQQNSWEPTGCCVWEHKCTEREGELRCLHGME
Ad40  178  LTFTSHKTFVEMMGRFLRAYVESYAGVKNNEWEPTGCAIWLHGCTEQEGVLRCYHGLE
Ad41  179  LTFTTQKTFTE LMGRFLAAYVETYAGVKHHNWDTTGCAVWAHGCTREEGVLRCFHGRE

Ad2   290  MINKEH VIEMDVTSENGQRALKEQ SSKAKI VKNRWGRNVVQISNTDARCCVHDAACPA
Ad4   269  MIQKEHMIEMDVASENGQRALKENPDRAKVTQNRWGRSVVQLANNDARCCVHDAGCAT
Ad5   290  MINKEH VIEMDVTSENGQRALKEQ SSKAKI VKNRWGRNVVQISNTDARCCVHDAACPA
Ad7   275  MIQKDHIIEMDVASENGQRAMKENPDRAKITQNRWGRNVVQLANNDARCCVNDANCAT
Ad12  245  MVRKEHLVEMDVTSESGQRALKENPSKAKVAQNRWGRNVVQIKNDDARCCFHDVGCGN
Ad40  236  MI QKEQLVEMDVASENAQRALKEHPSRAKVVQNRWGRSVVQLKNDDARCCVEDVSCAT
Ad41  237  MIQKEQVVEVDVGSENGQRALKEQPSKTKVVQNRWGRSVVQIKNDDARCCAEDVSCGN

Ad2   348  NQFSGKSCGMFFSEGAKAQVAFKQIKAFMQALYPNAQTGHGHL-LMPLRCECNSKPGH
Ad4   327  NQFSSKSCGVFFTEGAKAQQAFKQLEAFMKAMYPGMNADQAQVMLIPLHCOCNHKPGC
Ad5   348  NQFSGKSCGMFFSEGAKAQVAFKQIKAFMQALYPNAQTGHGHL-LMPLRCECNSKPGH
Ad7   333  NQFSSKSCGMFYTEGIKAQEAFKQYEAFMKAVYPGITPDQARMMLIPIHCDCNHKPGC
Ad12  303  NSFSGKSCGLFYSEGMKAQIAFRQI EAFMLADYPHMRHGQKRL-LMPVRCECLNKQDG
Ad40  294  NVFSAKSCGLFFSEGTKAQTAFLQI EAFMQAEYPKMQNGLKRL-LMVMRCDCLYKPTG
Ad41  295  NMFSSKSCGLFFSEGLKAQI AFKQMQAFLQAEYPQMQRGQQRI-LVPLRCECLNKKDL

Ad2   405  APFL GRQLPK LTPFA LSNAEDLDADLISD KSVLASVHHPALI VFQCCNPVYRNSRAQG
Ad4   385  VPTMGRQTCKMTPFGMANAEDLDVDGITDATVLASVKHPALMVFQCCNPVYRNSRAQN
Ad5   405  APFL GRQLPK LTPFA LSNAEDLDADLISD KSVLASVHHPALI VFQCCNPVYRNSRAQG
Ad7   391  APVMGRQTCKMTPFGMANAEDLDVATISDPTVLASVRHPALMVFQCCNPVYRNSRVQN
Ad12  360  LPRM GRQLCKI TPFN L SNVDNI DI NEVTDPGALASIKYPCL LVFQCANPVYRNARGN
Ad40  351  VPQLGROMCKATPFA LSN VDSLRAEEVTDKVALASIQYPCVLVYQ CANPVYRNSRGG
Ad41  352  VPQLGRQMCKVTPFA LSG AEDLK TSEVTDKSALASI LHPCVLVFQ CANPVYRNSRGS

Ad2   463  GGPNCDFKISAPDLLNALVMVRSLWSENFTE-LPRMVVPEFKWSTKHQYRNVSLPVAHS
Ad4   443  AGPNCDFKISAPDLLGALQLTRKLWTDSFPDTLLPKLLIPEFKWLAKYQFRNVSLPAGHA
Ad5   463  GGPNCDFKISAPDLLNALVMVRSLWSENFTE-LPRMVVPEFKWSTKHQYRNVSLPVAHS
Ad7   449  AGPNCDFKISAPDLLGALQLTRKLWQDTFPEIPVPKLVIPEFKWQNRLQFRNVSLPAGHY
Ad12  418  AGPNCDFKISAPDVMGALQLVRQLWGENF-DGSPPRLVIPEFKWHQRLQYRNISLPTNHG
Ad40  409  QGPNCDFKISAPDLLGALQLVRRLWGENV-DGPLPKMLIPEFKWSSRLQYRNVALPASHG
Ad41  410  AGPNCDFKISAPDVISALQLVRQFWKENV-EDPLPKLIIPEFKWSTRLQYRNVALPTGHG
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Fig.II-8. Comparison of tyrosine (bold) distribution in C-terminal of Ad DBPs. Four conserved regions are underlined (CR1:178-186; Zinc finger domain: 273-286; CR2:322-330; CR4: 464-475). (from Vos et al, 1988).

Based on the known specificity of the reagent, chemical modification of a protein is a simple method to identify the involvement in DNA binding of a particular amino acid (Bandyopadhyay and Wu, 1978). Under mild conditions, the reaction of iodine with protein modifies primarily tyrosines, leading to the iodination of the phenolic moiety in tyrosine and resulting in the loss of hydrophobic interactions between phenolic moiety of the tyrosine and the aromatic bases of the polynucleotide (Shamoo et al, 1987). When DBP was iodinated with KI_3 at molar ratios of KI_3 : DBP of 25:1, 50:1 & 100:1 (corresponding to molar ratio of KI_3 : Try in Ad2 DBP of 4:1, 8:1 & 16:1 respectively), the ssDNA binding activity of DBP was completely abolished (Fig.II-9), suggesting that these (accessible to modification) tyrosines of DBP were involved in ssDNA binding. Western blotting of untreated and iodinated DBP with MAb 12B8 showed (Fig.II-10) that the antigenic site and the size of the DBP had not been altered by this modification, suggesting that iodination under these conditions did not denature the protein and the antigenic site recognized by MAb 12B8 may be not located near these tyrosines. This is in agreement with the result that MAb (12B8) binding of DBP did not affect its DNA binding. Comparing previous results (see introduction 3.3), Try_{510} (conserved) is within the C-terminal 46 amino acids which has been shown to abolish DNA binding when deleted (Vos et al, 1988a) and Try_{455} (highly conserved) has been reported to be a third putative contact point with DNA by photochemical cross-linking (Cleghon and Klessig, 1992) and its location is very close to the conserved region 3 (CR3: 464-475) which has

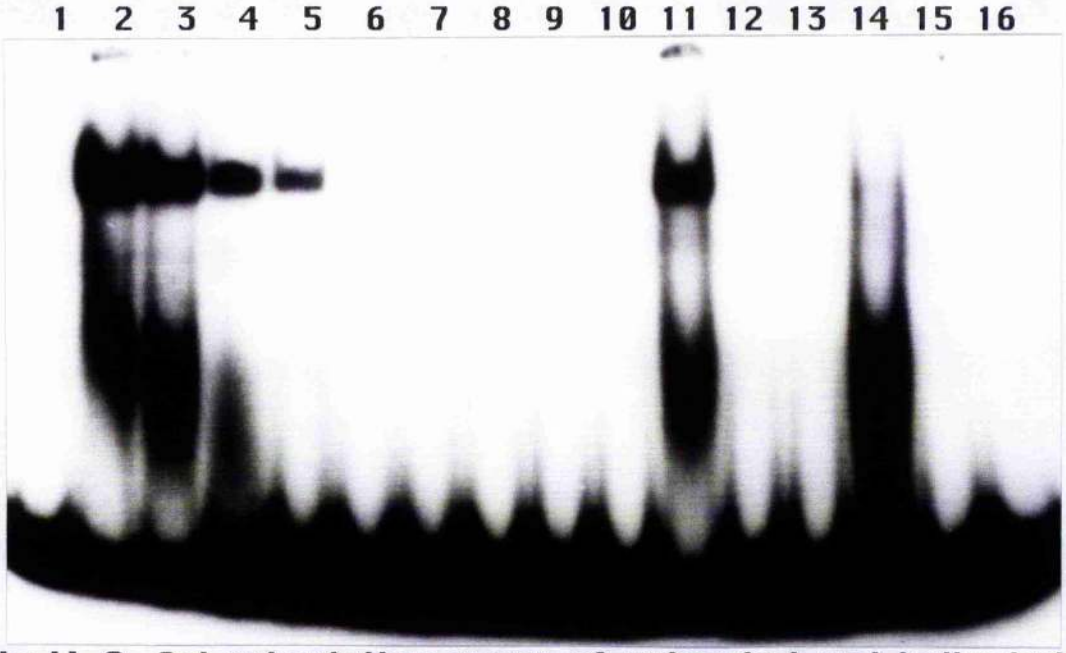

Fig.II-9. Gel retardation assay of untreated and iodinated DBP. Lane1: probe (^{32}P labelled ssDNA); Lanes2-5: 200ng, 100ng, 50ng & 25ng of untreated (control) e-DBP; Lanes6-8: 200ng, 100ng & 50ng of iodinated e-DBP (KI_3:DBP of 25:1); Lanes 9-10: 100ng of iodinated e-DBP (KI_3:DBP of 50:1 & 100:1); Lane11: 100ng of untreated l-DBP; Lane12-13: 100ng of iodinated l-DBP (KI_3:DBP of 50:1 & 100:1); Lane14: 100ng of untreated B-DBP; Lanes15-16: 100ng of iodinated B-DBP (KI_3:DBP of 50:1 & 100:1). e: early, DBP was purified from Ad2 infected Hela cells in the presence of 10mM Hydroxyurea; l: late, DBP was purified from Ad2 infected Hela cells in the absence of 10mM Hydroxyurea; B: DBP was purified from Baculo-virus.

been suggested to be involved in DNA binding by mutagenesis (Neale and Kitchingman, 1989; 1990). Thus, these two tyrosines are most likely to be involved directly in the interaction with DNA. Try_{380} is highly conserved but has not been reported to be related to DNA binding. Try_{251} and Try_{260} are not conserved but they are close to the Zinc finger which has been suggested to be required for DNA binding (Eagle and Klessig, 1992 and might be involved in DNA binding. Try_{195}

(highly conserved) is the putative tyrosine phosphorylation site and can be phosphorylated in vitro in a peptide. Try_{195} is located at the N-terminal side of C-terminal domain and is not far from Try_{251}, Try_{260} & Zinc finger. Phosphorylation and dephosphorylation on Try_{195} might therefore indirectly regulate the function of DBP in DNA binding in vivo.

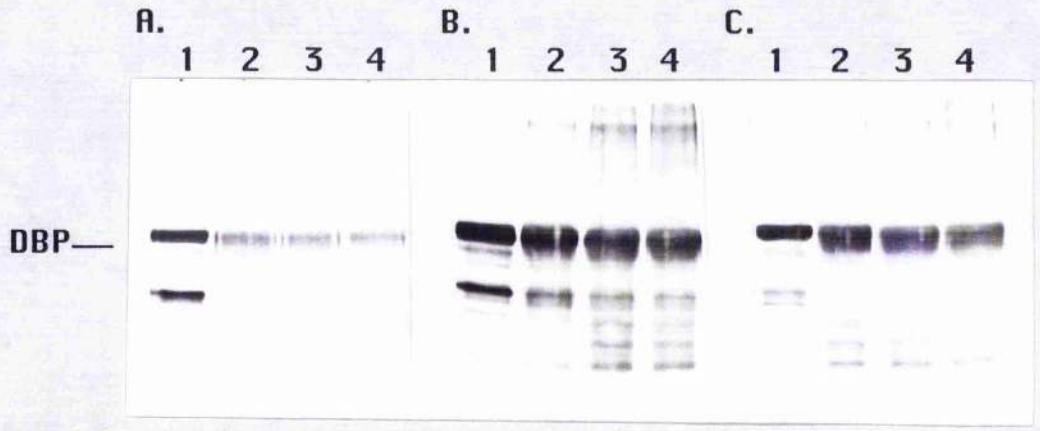

Fig.II-10. Western blotting of untreated and iodinated DBP. Lane1: untreated DBP; Lanes2-4: iodinated DBP (KI_3:DBP of 25:1, 50:1 & 100:1 respectively). A. early DBP; B. late DBP; C. Baculo-viral DBP.

3. Tyrosine kinases —— are they required for Ad2?

Tyrosine phosphorylation is very important for both cells and viruses due to its control in signal transduction and protein function. In normal cells, the tyrosine kinase activity is maintained at a certain level. When cells are transformed or infected by a virus, this level could be elevated since many oncoproteins are tyrosine kinases themself or possess a tyrosine kinase activity. In Ad2 infected Hela cells, no increase in activity of tyrosine kinase was detected using a

synthetic peptide substrate (RR-SRC). Are tyrosine kinases required by Ad2? A very simple experiment was carried out by immunofluorescence using a tyrosine kinase inhibitor Genistein. In this experiment, Hela cells were grown in multiwell slides and infected with Ad2 (20p.f.u/cell) as follows: (1) in the absence of Genistein & Hydroxyurea: (2) in the presence of Genistein (50μg/ml) but absence of Hydroxyurea; (3) in the presence of Hydroxyurea (10mM) but absence of Genistein. After 24h p.i, the cells were fixed, penetrated and detected for DBP and Hexon using MAbs to DBP and Hexon. In the case of (1), DBP and Hexon were detected in every cell (100%); in the case of (2), DBP was detected in every cell but Hexon was only detected in about 30% of the total cells; in the case of (3), DBP was detected (100%) but no hexon was detected (data not shown). DBP is an early event of Ad infection (it was synthesized before the onset of viral DNA replication) while the hexon is a late event (it was synthesized after the onset of viral DNA replication). When Ad2 DNA replication is blocked (e.g. in the presence of hydroxyurea), only DBP is produced. Genistein is an isoflavone compound originally isolated from the fermentation broth of Pseudomonas sp (Ogawara et al, 1986) and has been shown to be a highly specific inhibitor for tyrosine kinases (Akiyama et al, 1987). When Hela cells were infected with Ad2 in the presence of Genistein, DBP synthesis was not affected but the synthesis of hexon was partially inhibited. Would this imply a role of tyrosine phosphorylation in Ad2 DNA replication? Would this be related to tyrosine phosphorylation of DBP?

It has been known for quite some time that DBP is heavily phosphorylated early after infection and it has been proposed that the various phosphorylated forms of DBP may carry out the different roles of this multifunctional protein. However the role of phosphorylation of DBP is still a matter of conjecture. Mutation studies has suggested that the overall phosphorylation state of the protein may be important for its own expression (Morin et al, 1989). DBP is involved in Ad transcription. It interacts with E1A, E2A, E4 & MLP promoters, stimulating the transcription directed from these promoters (except E4) especially from MLP. Would it be possible that DBP may act as an acidic transcriptional activitor in transcription (Lin and Green, 1991) and its overall phosphorylation state facilitates this function (providing an acidic region to interact with other transcriptional factors)? Would it be possible that the phosphorylation state of DBP may be controlled by the phosphorylation/dephosphorylation of Try_{195} (which in turn may be controlled by a viral tyrosine kinase/phosphatase)? Early after infection, this tyrosine could be phosphorylated, contributing to the conformation that would be favoured by the interaction with transcriptional factors and promoters while late after infection , this tyrosine may be dephosphorylated, providing the conformation that would favoured by the interactions with replication proteins and DNA (the promoter of DBP gene is shifted when DNA replication started). It is difficult to say whether the reduced synthesis of hexon noted above is due to an inhibition of late gene transcription or a block of viral DNA synthesis. However, from this simple experiment, the role

that tyrosine kinases may play cannot be dismissed although there is no detectable increase in tyrosine kinase activity in an Ad2 infection.

Part III Expressing DBP in E coli using pGEX-2T vector

The technology of DNA manipulation in vitro has developed to the level that many eukaryotic genes can be expressed in prokaryotes such as *Escherichia coli* (E. coli) (Marston, 1986). The basic approach used to express foreign genes in E coli is to insert the genes of interest into an expression vector then transform the vector (containing inserted foreign genes) into E. coli (Hanahan, 1983). pGEX vectors are designed so that foreign polypeptides can be expressed in E coli as fusions at the C-terminus of sj26, a 26K glutathione S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum* (Smith and Johnson, 1988). The advantage of this system is that it allows expressed protein to be purified under non-denaturing conditions by affinity chromatography on immobilised glutathione. The pGEX-2T vector also contains sequences that encode protease cleavage sites specifically recognised by thrombin (Fig.III-1.(a)).

Methods:

1. Preparation of pGEX-2T plasmid

Growth of bacteria A colony of bacteria (strain JM101) transformed by plasmid DNA pGEX-2T (Pharmacia) was plated onto a Luria-Agar plate containing 100μg/ml ampicillin and incubated overnight at 37°C. One toothpick scrape of this was transfered into a 2L flask containing 500ml Luria-Broth (LB) together with 100μg/ml ampicillin and incubated overnight

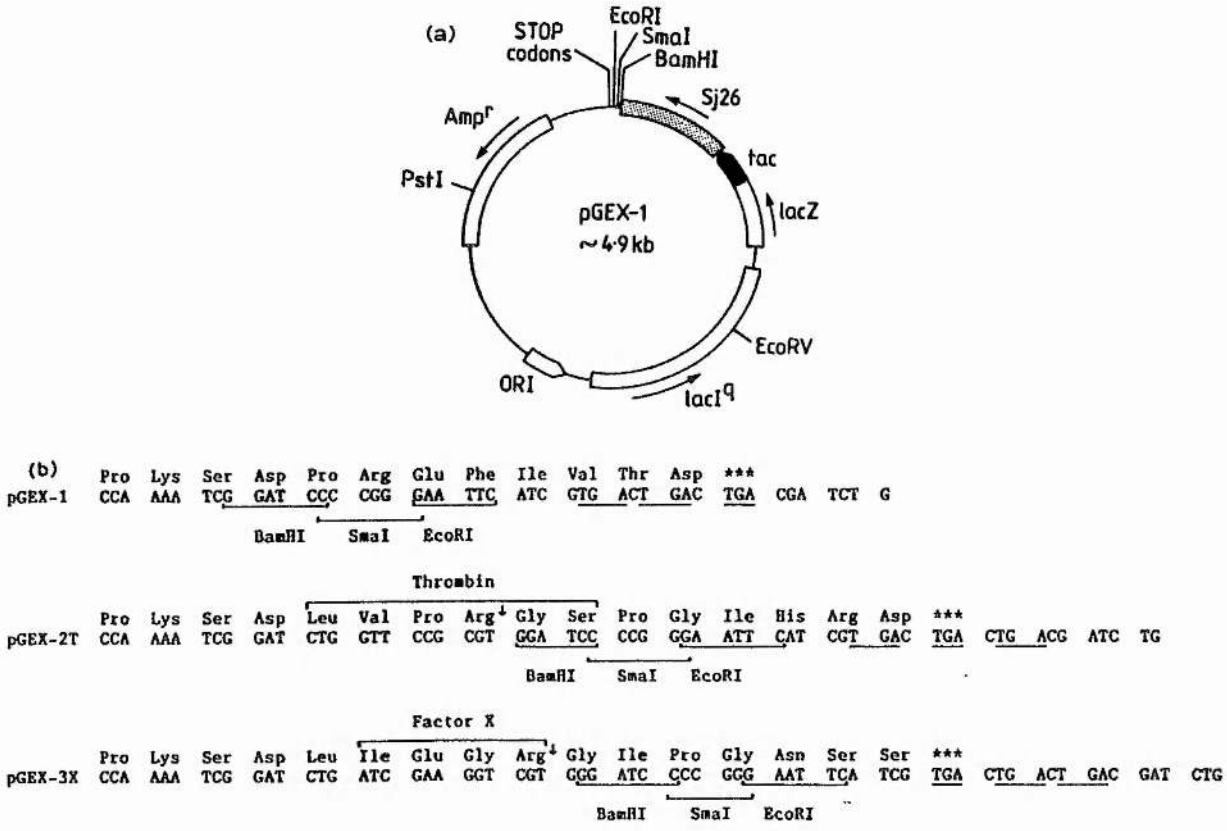

Fig.II-1. Structure of the pGEX vectors.(a). Schematic representation of pGEX-1. The positions of EcoRI, BamHI and PstI restriction sites are indicated. (b). Nucleotide sequence of pGEX-1, pGEX-2T and pGEX-3X at the C terminus of the Sj26 cDNA. The position of Thrombin cleavage sites of pGEX-2T is indicated (from Smith and Johnson, 1988).

in a shaker at 37°C, allowing cells to grow to stationary phase.

Lysis of bacteria by alkali The cells were pelleted in 2x250ml bottles by centrifugation at 8,000 rpm for 10 min at 4°C (Beckman JA14). Each of the pellets were then resuspended in 9ml of 50mM Glucose/25mM Tris-HCl (PH 8.0)/10mM EDTA followed by further addition of 1ml lysozyme (20mg/ml,

freshly made in 0.2M Tris-HCl, PH 8.0) and placed on ice for 5 min. After which, 20ml of 0.2M NaOH/1% SDS (freshly made from stock solution) was added and the mix was left on ice for a further 5 min followed by addition of 10ml 5M KAc pH 5.4. After 15 min incubation on ice, the mixture was clarified in 50ml tubes by centrifugation at 12,500 rpm for 40 min at 4°C (Beckman JA17). The supernatant was then filtered through two layers of cheese cloth and transferred to a fresh tube containing 15ml isopropanol. The mixture was left at room temperature for 15 min. Nucleic acid was then precipitated from the supernatant by centrifugation at 12,500 rpm for 5 min and the resulting pellet of plasmid DNA was washed with 70% ice cold ethanol, dried and resuspended in 1ml of TE buffer (10mM Tris-HCl, 1mM EDTA, PH 7.5).

Purification of crude plasmid DNA by equilibrium centrifugation in CsCl-Ethidium Bromide gradients. 1.2g of caesium chloride (CsCl, Sigma) was added to 1ml solution of crude plasmid DNA followed by the addition of 50µl of 10mg/ml Ethidium Bromide (EtBr, Sigma). The mixture was left in the dark at room temperature for 5 min and clarified by centrifugation at 12,000g for 3 min (MSE bench microcentrifuge). The supernatant was transferred to a 2ml Beckman polyallomer Quickseal tube, and the tube was topped up and balanced with TE/CsCl/EtBr and heat sealed. The plasmid DNA was centrifuged to equilibrium at 80,000 rpm overnight at 20°C (Beckman TL100) and visualised as a pink band. The plasmid band was removed by side puncture using 21G hypdermic needles.

Butanol extraction of EtBr Ethidium bromide was removed from the DNA solution by repeated extractions with an equal volume of n-Butyl-alcohol saturated with H_2O until the pink color had disappeared completely.

Isopropanol and Ethanol precipitation Two volumes of water was added to the DNA solution to dilute the CsCl and allow the DNA solution to mix with isopropanol, of which, 0.6-1 volume was added afterwards. After standing at room temperature for 20 min, the mixture was spun at 12,000g for 5 min (MSE bench microcentrifuge). The precipitated DNA was washed with 70% ice cold ethanol and resuspended in 400μl H_2O. 40μl (1/10 volume) of 3M NaAc (PH 4.8) and 1ml (2-3 volumes) of ice cold ethanol were added. The mixture was left at -20°C for 15 min and spun as before. The DNA pellet was then washed, dried, taken up in 400μl water and stored at -20°C.

2. Polymarase chain reaction (PCR)

DBP genes of whole length protein, N-terminal domain (amino acids 1-173) and C-terminal domain (amino acids 174-529) were amplified by polymerase chain reaction (PCR) (Saiki et al, 1988) using a DNA thermal cycler (Techne Programmable Dri-Block PHC-1).

Primers Four oligonucletides, whose sequences were selected from Ad2 DBP genes (Fig II-2), were synthesized in an Applied Biosystems Model 381A DNA synthesiser to form three pairs of primers used in PCR. For whole length protein, upstream primer is 5'-GCG CGG ATC CCG GGA AGA GGA GCA GCG CGA AAC C-3' and downstream primer is 5'-GCG CGA ATT CCC CCC CAC CCT TGC CGT CTG CGC CGT T-3'; For N-terminal protein, upstream primer

M A S R E E E Q R E T T P E R G R G A A R R P P T	25
M E D V S S P S P S P P P P R A P P K K R L R R R	50
L E S E D E E D S S Q D A L V P R T P S P R P S T	75
S T A D L A I A S K K K K K R P S P K P E R P P S	100
P E V I V D S E E E R E D V A L Q M V G F S N P P	125
V L I K H G K G G K R T V R R L N E D D P V A R G	150
M R T Q E E K E E P S E A E S E S T V I N P L S L	175
P I V S A W E K G M E A A R A L M D K Y H V D N D	200
L K A N F K N L P D Q V E A L A V C K T W L N E	225
E H R G L Q L T F T S K K T F V T M M G R F L Q A	250
Y L Q S F A E V T Y K H H E P T G C A L W L H R C	275
A E I E G E L K C L H G S I M I N K E H V I E M D	300
V T S E N G Q R A L K E Q S S K A K I V K N R W G	325
R N V V Q I S N T D A R C C V H D A A C P A N Q F	350
S G K S C G M F F S E G A K A Q V A F K Q I K A F	375
M Q A L Y P N A Q T G H G H L L M P L R C E C N S	400
K P G H A P F L G R Q L P K L T P F A L S N A E D	425
L D A D L I S D K S V L A S V H H P A L I V F Q C	450
C N P V Y R N S R A Q G G G P N C D F K I S A P D	475
L L N A L V M V S L W S E N F T E L P R M V V P E	500
F K W S T K H Q Y R N V S L P V A H S D A R Q N P	525
F D F	

Fig.II-2. Amino acid sequence of Ad2 DBP (from Russell et al, 1989).

is 5'-5'-GCG CGG ATC CCG GGA AGA GGA GCA GCG CGA AAC C-3' and downstream primer is 5'-GCG CGA ATT CTT ATA CGG CCG CCA GAG

CTT CCA CTT GG-3'; For C-terminal protein, upstream primer is 5'-GCG CGG ATC CAG CCT GCC GAT CGT GTC TGC GTG G-3' and downstream primer is 5'-GCG CGA ATT CCC CCC CAC CCT TGC CGT CTG CGC CGT T-3'. EcoRI (GAATTC) and BamHI (GGATCC) sites were underlined.

Reaction mixes PCR mixes contained 50mM KCl, 10mM Tris-HCl (pH 8.4), 1.5mM $MgCl_2$, 200μM each of the four dNTPs, 100ng of Ad2 DNA, 1μM of each primer and 2 units of Taq DNA polymerase (Gibco) in a total volume of 100μl.

Thermal cycle parameters These were 94°C, 1.5 min for denaturation; 50°C, 2 min for annealing; 72°C, 3 min for elongation and a total of 30 cycles. A condition of 94°C, 10 min was incorporated at the beginning of the cycle for initial denaturation of the template and 72°C, 5 min was used in final extension.

3. Inserting DBP gene into pGEX-2T vector

The plamid DNA of pGEX-2T and DBP DNA fragments of PCR products were double cleaved with restriction enzymes EcoRI and BamHI, isolated by agarose gel electrophoresis, recovered from the gel and joined together using T4 DNA ligase.

Restriction endonuclease digestion Each digestion was carried out in the conditions recommended by the manufacture for individual enzymes. The reaction mix contained 2-10μg of DNA, 1-5μl (8-10 units/μl) of enzymes (Gibco) and 1/10 volumes of 10x buffer supplied by the manufacture in a total volume of 10-50μl and was incubated at 37°C for 1 hour.

Agarose gel electrophoresis (Maniatis et al, 1982) All DNA samples were run on mini gels containing 0.8-1% (w/v) agarose in 1x TAE buffer (0.04M Tris-Acetate/0.002M EDTA, pH 8.0) at 40mA of constant current for 3-4 hours, and visualised under long wave UV light. Prior to loading the sample, a 1/10 volume of dye mix containing 0.25% bromophenol blue, 0.25% xylene cyanol and 25% Ficoll (type 400) in water was added, and 5-10 min before visualising the DNA bands, ethidium bromide (10mg/ml in water) was added to running buffer (1xTAE) to a final concentration of 1μg/ml.

Recovery of DNA from agarose gel The DNA band of interest was cut out from agarose gel under UV light and mashed up in an Eppendorf tube. Phenol/chloroform was added just to cover the agarose and the mixture was mixed well using a flat toothpick and left at -70°C for more than 15 min. Immediately taken from -70°C, the mixture was spun at high speed (12,000g) in bench microcentrifuge for 5 min. The phenol/chloroform phase was removed carefully from the bottom. Spun briefly, the supernatant was transferred to a fresh tube and extracted by ether 2-3 times to remove the trace of phenol as follows: equal volume of ether was added and mixed by vortex and the top phase (ether) was removed and discarded. The DNA was recovered by ethanol precipitation as described before.

Ligation DNA fragments of pGEX-2T (vector) and DBP (insert) were ligated in a mixture of 10μl containing 0.5μl of T4 DNA ligase (Gibco) and 2μl of 5x ligase buffer (Gibco). The reaction was carried out at 12°C overnight (12-16 hours). The molar ratio of vector DNA to insert DNA is about 1:3.

4. Introducing recombinant plasmid into E. coli

Preparation of competent cells An overnight culture of E. coli cells (strain JM101) was diluted at 1:100 into 100ml Luria-Broth in 500ml flask and incubated at 37°C with vigorous shaking until the cells reach an OD_{600} of 0.3-0.4. The cells were pelleted by centrifugation at 1000g for 10 min at 4°C and resuspended in 10ml (1/10 original volume) of ice cold 1x TSS (Chung et al, 1989). The TSS was Luria-Broth containing 10% (w/v) PEG-400 (polyethylene glycol Wt.400, DBH), 5% (v/v) DMSO (dimethyl sulfoxide, Sigma), and 50mM $MgCl_2$ at a final pH of 6.5. Competent cells were used immediately or left on ice for up to 24 hours or stored in TSS at -70oC. Frozen cells were thawed on ice and used immediately.

Transformation 0.1μg-0.5μg of plasmid DNA or 5μl of ligation mix was added into 0.1ml competent cells. The mixture was incubated on ice for 30 min with occasional gentle mixing and heat shocked at 42°C for 90 sec. After this, the mixture was transferred into a 10ml universal containing 0.9ml of Luria-Broth with 20mM glucose and incubated in a 37°C shaker for 1 hour. 0.1-0.2ml of the above mixture was then plated onto a Luria-Agar plate containing 100μg/ml ampicillin and grown at 37°C for 18-20 hours.

5. Screening of transformants

Recombinant clones were selected and grown in Luria-Agar containing 100μg/μl ampicillin in a plate at 37°C. Their DNA was isolated by rapid boiling method (Holmes and Quigley, 1981) and analysed by restriction endonuclease digestion.

STET boiling method of mini prep. One toothpick scrape of recombinant colony culture was transferred to an Eppendorf tube. 200μl of STET buffer (8% sucrose/5% Triton X-100/50mM EDTA/50mM Tris-HCl, PH 8.0) and 16μl of lysozyme (10mg/ml fresh made in water) were added. The mixture was well mixed using a toothpick and left at room temperature for 5 min. After which, the tube was placed in a boiling water bath for 1 min and immediately spun at 12,000g for 5 min (MSE bench microcentrifuge). The pellet was removed using a toothpick and 150μl of isopropanol was added. The mixture was mixed by vortex and left at room temperature for further 10 min. Plasmid DNA was then precipitated by centrifugation at 12,000g for 5 min, washed, dried, and resuspended in 40μl of water. 5μl of the solution was used in each digestion.

6. Purification of the fusion protein

100ml of overnight cultures of E. coli transformed with parental or recombinant pGEX-2T plasmids was added to 900ml (1:10 dilution) of fresh medium and grown at 37°C with shaking until cells reach an OD_{600} of 0.6. Then 1ml of 0.1M IPTG (Isopropyl-β-D-thiogalactopyranoside, Sigma) was added to a final concentration of 0.1mM and the cultures were incubated at 25°C with shaking for a further 3 hours. The cells were pelleted and lysed by sonicating (MSE ultrasonic probe) on ice in 10ml (1/10 volume) of PBS containing 0.5M NaCl, 1mM PMSF and 1% Triton X100. The lysate was sonicated 8-16 times at 30 sec/time and clarified by centrifugation at 31,400g for 30 min at 4°C (Beckman, JA20). The supernatant

was saved and mixed with glutathione-agarose beads (Sigma) for 2 hours at 4°C by continuous rotation. The glutathione agarose beads were previously swollen in PBS and washed with PBS alone first, PBS with 1% Triton X100 afterwards. The mixture was loaded onto a column and the column was washed with PBS. The fusion protein was then eluted from column by 15mM reduced glutathione (Sigma)/0.5M NaCl/50mM Tris-HCl, pH 8.0 in 1ml fractions.

Results:

1. Construction of a plasmid containing DBP genes

DBP genes of whole length, N-terminal and C-terminal were selected, amplified and inserted into pGEX-2T vector as described in methods section. The recombinant plamids were introduced into E. coli (Strain JM101) and the transformants were screened for insertion of DBP genes. Using mini prep STET method, the recombinant DNA was isolated. Double digestion pGEX-2T with EcoRI and BamHI generated one big fragment about 5.6K in agarose gel (Fig.II-3. A. Lane 6). While using BamHI and PstI, double digests of pGEX-2T generated one big fragment (5.6K) and one small fragment about 1.0 K in agarose gel (Fig.II-3. B. Lane 12 and C. Lane 6). Calculated from gene sequence, the molecular weight of three DBP inserts whole length, N-terminal and C-terminal are 1.6K, 0.656K and 1.09K respectively. If a recombinant plasmid contains an insert of whole length DBP gene, double digesting the plasmid with EcoRI and BamHI would generate a fragment of 1.6K. Fig.II-3. A. Lanes 1-5 showed double digests of

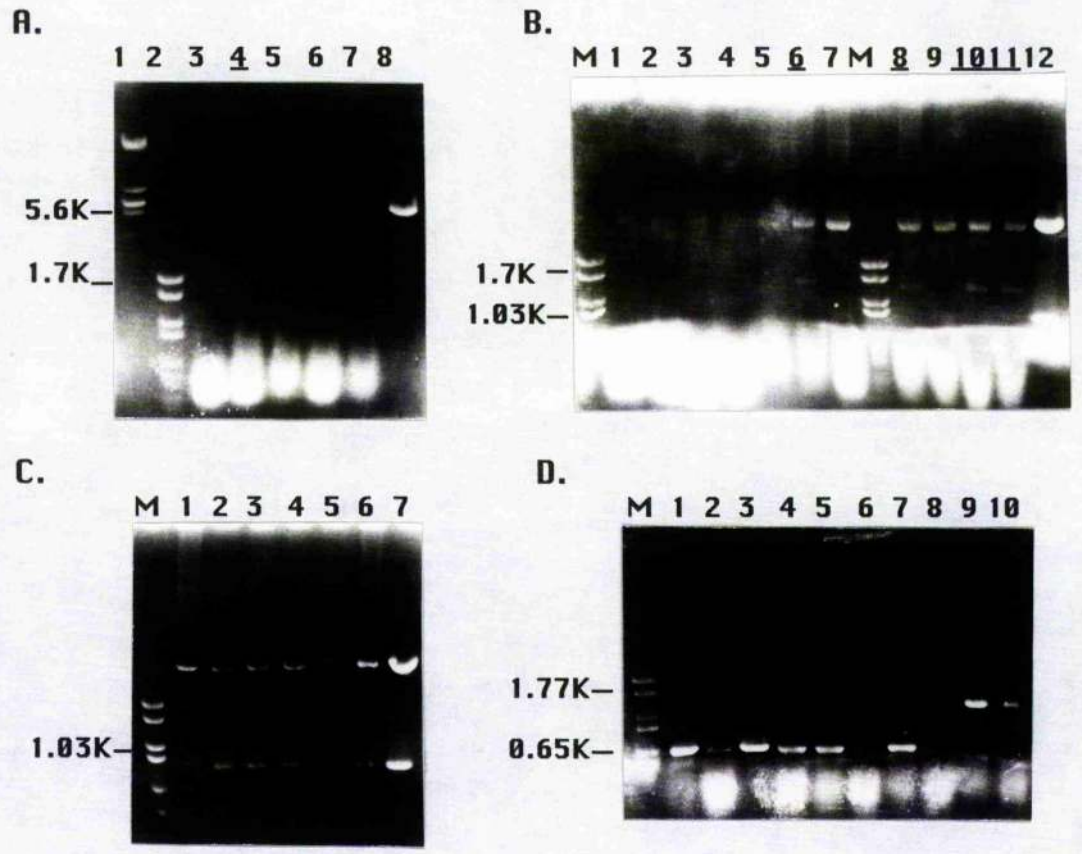

Fig.II-3. Construction of a plasmid containing DBP gene. A. EcoRI/BamHI double digests of recombinant DNA of pGEX-2T inserted with whole length DBP gene (lanes 1-5) and pGEX-2T (lane 6). Molecular weight markers : 21.2K, 7.4K, 5.8K, 5.6K, 4.8K and 2.18K, 1.77K, 1.23K, 1.03K and 0.65K. B. BamHI/PstI double digests of recombinant DNA of pGEX-2T inserted with N-terminal DBP gene (lanes 1-11) and PGEX-2T (lane 12). C. BamHI/PstI double digests of recombinant DNA of pGEX-2T inserted with C-terminal DBP gene (lanes 1-5) and PGEX-2T (lane 6). D. PCR products of whole length DBP gene (lane 9 and 10) used template from recombinant plasmid DNA of lane 4 (Fig.II-3. A) and N-terminal DBP gene (lanes 1-8) used template from recombinant plasmid DNA of lanes 6, 8, 10 and 11 (Fig.II-3. B).

recombinant plasmids with EcoRI and BamHI. A band appeared in lane 4 was just below the weight marker of 1.77K, suggesting that the whole length DBP gene (1.6K) was

inserted in this plasmid. This was confirmed by a PCR reaction using the recombinant plasmid DNA containing an insert of whole length DBP gene (shown in lane 4) as a template (Fig.II-3. D. Lane 9 and 10). Double digests of a recombinant plasmid with BamHI and PstI would generate a small fragment of 1.65K (1.0K+0.65K) for N-terminal insert and a 2.09K (1.0K+1.09K) fragment for C-terminal insert. Fig.II-3. B. Lanes 6, 8, 10 and 11 showed a band with an expected size of about 1.65K, suggesting an insert of N-terminal DBP gene (0.65K) in these plasmids. This was also confirmed by a PCR reaction using these plasmids DNA as templates (Fig.II-3. D. Lanes 1-8). Fig.II-3. C. Lanes 1-5 showed a band about 1.03K, suggesting that there were no inserts in these plasmids since an expected small fragment generated from double digests with BamHI and PstI would be 1.65K for a recombinant plasmid containing N-terminal insert and 2.09K for a recombinant plasmid containing C-terminal insert.

2. Production of expressed DBP in E. coli

An overnight culture of bacterial cells transformed with pGEX-2T alone or pGEX-2T inserted with whole length DBP gene or N-terminal of DBP gene was diluted 1:10 in fresh medium, grown at 37°C to OD_{600}=0.6 and IPTG added to 0.1mM. Samples were taken after 3 hours induction and separated by 16% SDS PAGE followed by staining with coomassie blue. GST was clearly present in gel after induction (Fig.II-4. A. Lane 1) but fusion proteins were not (Fig.II-4. A. Lanes 3 and 5). By reducing the temperature of induction, a fusion protein was demonstrated in a western blot (Fig.II-4. B. Lane 5). Using

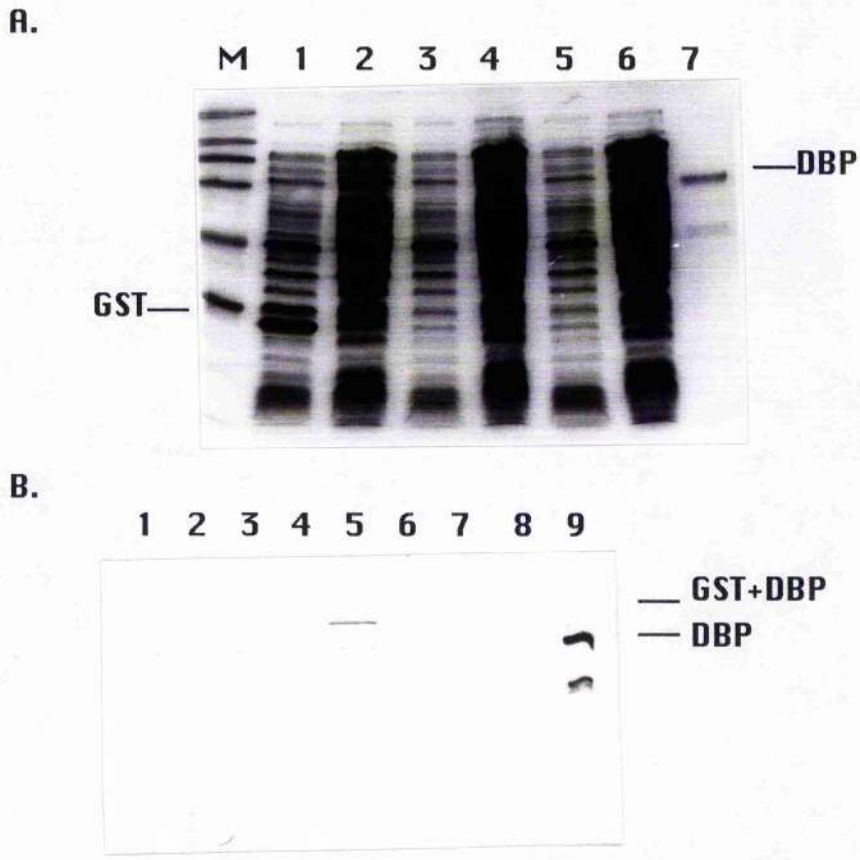

Fig.II-4. Production of expressed DBP in E. coli (western blotting): A. Expression and induction of fusion proteins. Lane 1: pGEX-2T with IPTG; Lane 2: pGEX-2T without IPTG; Lane 3: recombinant pGEX-2T/N-terminal insert with IPTG; Lane 4: recombinant pGEX-2T/N-terminal insert without IPTG; Lane 5: recombinant pGEX-2T/whole length insert with IPTG; Lane 6: recombinant pGEX-2T/whole length insert without IPTG; Lane 7: purified DBP. B. The effect of temperature on induction. Lanes 1 & 2: pGEX-2T induced at 25°C & 37°C; Lanes 3 & 4: pGEX-2T mock-induced at 25°C & 37°C; Lanes 5 & 6: pGEX-2T/Whole length insert induced at 25°C & 37°C; Lanes 7 & 8: pGEX-2T/Whole length insert mock-induced at 25°C & 37°C.

different concentration (0.1-1mM) of IPTG and different induction time (1-6 hours), these results have not been improved (data not shown).

Discussion:

In order to ascertain the relationship between phosphorylation status and various functions of DBP, it would be very useful to have the protein expressed totally devoid of phosphorylation, since bacterial protein kinases apparently do not catalyze the phosphorylation of exogenous proteins (Rickenberg and Leichtling, 1986) and protein kinases in prokaryotes are not as prevalent as in eukaryotes. Thus expressing DBP in bacteria could be a possible way to get an unphosphorylated DBP. There is an example (Barik and Banerjee, 1991) that a phosphoprotein synthesized in E. coli also lacks phosphorylation but can be phosphorylated in vitro and is also functionally active in vitro. Apart from this, bacterial expression would provide a quick and inexpensive system in which mutagenesis can by carried out via PCR (Ho et al, 1989). All these considerations would be facilitated by the expression of DBP in E. coli. Unfortunately, although the DBP gene was inserted into pGEX-2T vector (Fig.II-3) and the recombinant plasmid was introduced into E. coli, the production of fusion protein was very low. The reason for that is currently unclear. Eukaryotic proteins expressed in E. coli are not properly modified (Schein, 1989), and bacterial cytoplasm is less stable in pH and higher in salt than eukaryotes. These probably would affect the expression of certain eukaryotic proteins. Because proteins expressed in bacterial are not properly folded this probably makes them less resistant to bacterial proteases.

Conclusions:

1. Two antibodies anti-Ptyr (phosphotyrosine) polyclonal antibody (PAb) and anti-DBP (Ad2 DNA-binding protein) monoclonal (MAb) were made and used in a study of DBP phosphorylation.

Anti-Ptyr PAb recognized phosphotyrosine-containing protein EGFR in fixed cells and crude cell extracts by immunofluorescence, immunoprecipitation and western blotting but failed to detect Ptyr in DBP in these assays. This could possibly be due the limitation of the assay system (including the anti-Ptyr itself) and the very low level of Ptyr in the protein.

Anti-DBP MAb possibly recognized one major linear antigenic site of DBP. Binding to this MAb apparently did not affect the binding of DBP to DNA while binding of DNA apparently affected the binding of DBP to this MAb and the stability of the complex of DBP-MAb. Probably binding of DNA to DBP induces a significant change in conformation resulting in the unavailability of the epitope.

2. DBP was not phosphorylated in an in vitro phosphorylation system under the conditions stated above, but a peptide containing the putative tyrosine phosphorylation site of DBP and its surrounding amino acids was shown to be phosphorylated in vitro. This implicated a potential of DBP to be phosphorylated at tyrosine and a possibility that DBP could be tyrosyl-phosphorylated in vivo under certain conditions.

Probably in an in vitro situation, the conformation of DBP was such that the phosphorylation of tyrosine was not accessible.

Iodination of DBP abolished DNA-binding activity of DBP but did not alter its antigenicity and apparent molecule size, suggesting an involvement of tyrosine residues in DNA binding of DBP and a possibility that tyrosine-phosphorylation of DBP might indirectly regulate its DNA-binding activity by altering its local conformation (or environment).

Using synthetic peptide substrates, no tyrosine kinase activity was detected in Ad2 infected Hela cells. However when Hela cells were infected in the presence of Genistein, a highly specific inhibitor of tyrosine kinases, the synthesis of hexon (a late protein) was reduced, suggesting that tyrosine kinase might be required for Ad2 viral DNA synthesis.

3. An attempt to express DBP in E.coli was also made in order to carry out oligonucleotide-directed mutageniseis. The DBP gene was inserted into pGEX-2T vector and transformed into E.coli, but the production of DBP in E.coli was very low.

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